American Society of Biological Chemists

Symposium on Amino Acid Metabolism[1]

Chairman: JOSEPH S. FRUTON

CATALYTIC ACTION OF THE METAL PEPTIDASES[2]

EMIL L. SMITH

From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah School of Medicine

SALT LAKE CITY, UTAH

PROTEIN metabolism in the animal begins with proteolysis. The proteolytic enzymes of the gastro-intestinal tract and other tissues hydrolyze the peptide bonds of the proteins to form peptides of varying size and finally free amino acids. It is customary to classify the proteases in two groups: the true proteinases which can attack the high molecular weight proteins and the peptidases which can attack the smaller units, the peptides. Thanks to the work of Bergmann, Fruton and their collaborators (1) we know that this classification is not entirely valid. These investigators have shown that proteinases can hydrolyze simple peptide derivatives. Thus, trypsin attacks such a simple compound as alpha-benzoyl-L-arginin-amide at the amide linkage. A more valid classification was initiated by these investigators (1) in distinguishing between the endopeptidases and the exopeptidases. This means that the endopeptidases can attack at the interior of completely or partially substituted peptide chains while the exopeptidases generally attack peptides or their derivatives at bonds adjacent to a free alpha-amino or alpha-carboxyl group or both. Hence the terminology which distinguishes between aminopeptidases, carboxypeptidases and dipeptidases. Both the endo- and exopeptidases show a marked specificity toward peptide bonds involving particular amino acids. For example, pepsin attacks compounds like carbobenzoxyglutamylphenylalanine or carbobenzoxyglutamyltyrosine. The only known compounds which pepsin can attack contain the aromatic rings of tyrosine or phenylalanine residues. Similarly, we speak of leucine aminopeptidase since this enzyme has a markedly rapid action on leucine peptides.

Most investigators agree that enzymes must form definite chemical compounds with their substrates. This thesis, first clearly stated by Michaelis and Menten (2), has been demonstrated by kinetic studies on a large number of enzymes. Keilin and Mann (3) found that peroxidase forms a complex with hydrogen peroxide, and, recently, Chance (4) has studied the spectroscopically well-defined compounds that are formed by catalase and peroxidase with their substrate, hydrogen peroxide. In 1926, Euler and Josephson (5) suggested that the polar groups which are required in the substrates of the peptidases indicate that the enzyme combines with its substrate not only at the sensitive peptide linkage but also with the free polar groups of the substrate; they spoke of this as the di-affinity concept. In other words, the enzyme has a two-point combination with its substrate.

Some years ago, Johnson and his co-workers (6) made the extremely important observation that many of the peptidase activities obtained from animal and plant tissues were greatly increased by the addition of certain metal ions. For example, it was found that leucine aminopeptidase is strongly activated by manganese or magnesium ions. It now appears that the rôle of the metals in the various peptidase systems is to form the complex between the proteins and the

[1] Detroit, Michigan, April 20, 1949.

[2] Much of the original work reported in this paper by the author and his collaborators was aided by grants from the United States Public Health Service.

B & Z Library

substrates. Evidence of this comes in part from the essentiality of the metal. Leucine aminopeptidase and other peptidases are inhibited by such typical metal poisons as cyanide, sulfide etc. It is also demonstrated by the fact that when the enzyme is purified the activity decreases unless metal ions are added back to the system (7).

The metal does not act simply as an activator of the enzyme but appears to be linked in true chemical combination with the protein of the whole enzyme system. This may be illustrated by the studies on purified leucine aminopeptidase. When protein, metal and substrate are added simultaneously, the activity is, at first, quite low

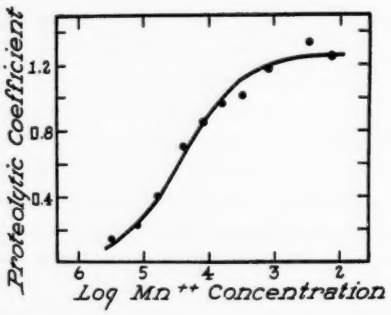
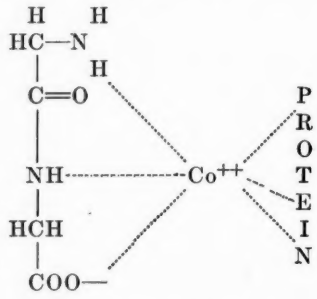


FIG. 1. COMBINATION OF LEUCINE AMINOPEPTIDASE AND Mn^{++}. The curve is the theoretical one from the mass law equation for combination of one Mn^{++} per active group of enzyme (8).

with slow hydrolysis of the substrate; this is followed by a rapidly increasing rate of reaction. In contrast to this, if the metal is allowed to stand with the protein for some time before its addition to the substrate, then it is found that the hydrolysis of the substrate proceeds quite rapidly following the usual kinetics of a first-order reaction. The occurrence of such a time-reaction between the metal and the protein must be interpreted as due to formation of a new compound (7). Similar time-reactions have also been found with many other enzymes, e. g., various peptidases, phosphatase and arginase.

If a complex is formed, then the combination between metal and protein should follow the usual mass action law for such a dissociable complex. The effect of Mn^{++} on leucine aminopeptidase does show the type of curve to be expected for the mass law equilibrium (8). The theoretical curve which is drawn through the data in figure 1 indicates the requirement of one atom of manganese for each active group of the enzyme.

An example of how the metal is bound with its substrate can be shown with the enzyme which has been called glycylglycine dipeptidase (9). From present information it appears that this enzyme is exceedingly specific; it is known to attack only this one peptide, glycylglycine. This enzyme is strongly activated by the addition of cobaltous ions and a definite spectroscopic complex is formed between glycylglycine and cobalt. This suggested that the enzyme-substrate complex might be formed by combination of the metal with the substrate on one hand and with the protein on the other. Therefore, many compounds were tested both for their ability to be hydrolyzed by the enzyme and for their ability to form complexes with cobalt. The structures are indicated in the following diagram.



If one hydrogen on the free amino group is replaced with a methyl group to form the compound sarcosylglycine, there is complex formation between the substrate and the metal and this compound is susceptible to hydrolysis. If, however, both hydrogens on the amino group are replaced to make N-dimethylglycylglycine, this compound is not hydrolyzed by the enzyme nor does it form a complex with cobalt. If the hydrogen at the sensitive peptide bond is substituted by a methyl group to make glycylsarcosine, there is no complex formation with cobalt and no hydrolysis by the enzyme. If the carboxyl group is altered to make glycylglycinamide, a spectroscopic compound is formed with cobalt but there is no hydrolysis by the enzyme. The complex formed between glycylglycinamide and cobalt has its maximal absorption position shifted about 40 mμ as compared to that given by cobalt and glycylglycine. It is clear that the chelation, the combination between cobalt and glycylglycinamide, must be different from the combination between cobalt and the substrate glycylglycine. This marked parallelism has also been found with a number of other compounds but they cannot all be mentioned here. Perhaps one more would be of

interest in this connection. The compound β-alanylglycine is not hydrolyzed by the enzyme nor does it form a spectroscopic complex with cobalt. It is well known that cobalt tends to make chelate compounds, that is ring compounds in which there are only five members, and that is the type of complex given by glycylglycine and cobalt. With β-alanylglycine a six-membered ring would be necessary but such compounds are very rare in the cobaltous series.

It appears from this, as a first approximation, that the ability of a compound to be susceptible to the action of this enzyme requires that the compound can form a complex with cobalt. Obviously, this is a necessary condition but not a sufficient condition since many peptides, for example, L-leucylglycine, can form complexes with cobalt but these compounds are not hydrolyzed by this enzyme. On the other hand, such a compound as glycyl-L-leucine, the isomeric peptide, does not form a strong complex with cobalt. Thus, there is exhibited the remarkable specificity of a metal ion in forming a strong complex with leucylglycine and a poor complex with glycylleucine. This specificity is indeed at a very low level. While we are all familiar with the concept that proteins are extremely specific in their combination with various substances, it is interesting to find such a specificity at the dipeptide level.

From the probable mode of combination of metal and substrate, we can begin to understand the differences between a dipepidase and an aminopeptidase. Glycylglycine dipeptidase requires in its substrate a free amino group, a free hydrogen at the peptide nitrogen and a free carboxyl group; it appears that cobalt forms a complex with three valences bound to the substrate and probably with three valences bound to the protein.[3] On the other hand, with leucine aminopeptidase it must be assumed that the metal, either manganese or magnesium, combines only with the free amino group and with the nitrogen at the peptide bond. A free carboxyl group is not required since the enzyme can hydrolyze leucinamide just as rapidly as it can the dipeptide, leucylglycine (7). Thus, we have an explanation in terms of the metal binding with these substrates of the difference between an aminopeptidase and a dipeptidase.

If the general thesis is correct that the exopeptidases require free polar groups in their substrates because the metal combines with these polar groups, then we must examine carboxypeptidase. This enzyme was first isolated in crystalline form by Anson (10) some years ago, but no attempt appears to have been made to ascertain whether a metal is present. It was found (11) that carboxypeptidase is strongly inhibited by such typical metal poisons as cyanide, sulfide, pyrophosphate, orthophosphate, citrate, oxalate etc. There is, therefore, a strong indication that a metal is present in carboxypeptidase. Examination, in a sensitive spectrograph, of the ash of a large amount of crystalline carboxypeptidase showed that the only metal present in significant amounts was magnesium. It was extremely gratifying that those metal ions that were used during the purification of the enzyme could not be detected in the final ashed material.

The first part of the thesis is confirmed in the sense that these exopeptidases, or the majority of them, appear to be metal proteins. Unlike the substrates for aminopeptidases and dipeptidases, the substrates for carboxypeptidase do not contain free amino groups. The best substrates for carboxypeptidase are those in which the amino group is blocked by an acylating group (12). For example, a typical substrated is carbobenzoxyglycyl-L-phenylalanine. The terminal carboxyl group is essential for the action of the enzyme. The peptide hydrogen is not essential for the action of this enzyme as is shown by the fact that the enzyme can hydrolyze such a compound as carbobenzoxytryptophylproline (13). Since proline is an imino acid, there is no hydrogen at the peptide bond. The unessential character of the peptide nitrogen is shown very strikingly by the discovery of Snoke, Schwert and Neurath (14) that carboxypeptidase can hydrolyze esters of the correct configuration; such a compound as hippurylphenyllactic acid is hydrolyzed by carboxypeptidase. Therefore, if it is assumed that the metal combines with the substrate in carboxypeptidase, the metal must bind with the free carboxyl group and with the keto group at the peptide bond and not with the nitrogen at all. An interesting correlation appears from this in that the chelate rings formed by the metal with the substrate in each case involve linkages at opposite sides of the sensitive peptide bond (15). It would

[3] It had previously been assumed that Co^{++} possessed a coordination number of four in the enzyme-substrate complex (9). A more reasonable description of the data is given by assuming that Co^{++} is bound in a six-coordinate complex.

appear that the explanation of the distinction between the three kinds of exopeptidases that have been discussed is in terms of the nature of the groups bound by the metal in each enzyme.

Since the metal in carboxypeptidase is tightly bound, it becomes possible to do quantitative inhibition studies in order to determine how many valences of the metal are available for combination with the substrate or, alternatively, for combination with an inhibitor which binds with the metal. It was found that each active group of carboxypeptidase is capable of binding two cyanide atoms or one orthophosphate or one sulfide.

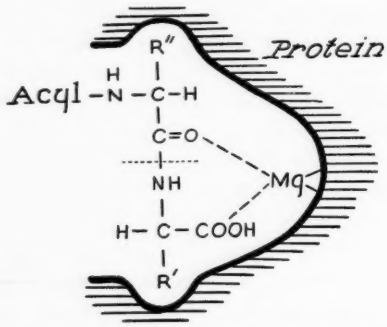
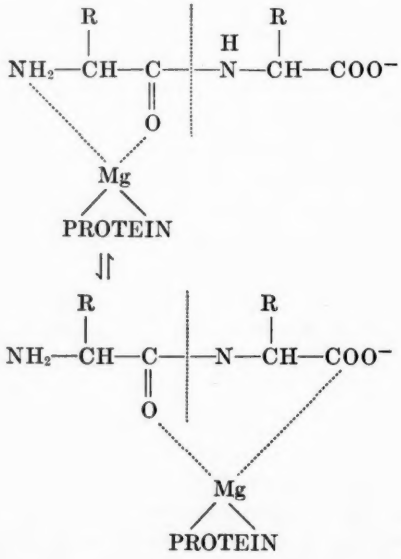


Fig. 2. DIAGRAM INDICATES SPATIAL RELATIONSHIPS of the binding of an acylated dipeptide by carboxypeptidase.

From these data it would appear that there are two free valences available for combination with the substrate or with an inhibitor (11, 15). This, then, is the true di-affinity as first defined by Euler and Josephson, but it may now be ascribed to the characteristic binding with the metal in the enzyme.

Obviously, in addition to the binding of the substrate by the metal through the polar groups, there are additional binding forces exerted by the protein moiety of the enzyme through the side chains of the substrate. For example, carbobenzoxyglycylglycine is a poor substrate for carboxypeptidase, but carbobenzoxyglycylphenylalanine is an excellent substrate (12). This indicates that the protein of carboxypeptidase binds very strongly with the terminal phenyl residue, and we can speak of this as the R group binding by the protein.

The polyaffinity concept first suggested by Bergmann and his co-workers (16) indicates that there must be a correct steric relationship between the polar groups of the substrate and the side chain branches of the substrate. Hence, carboxypeptidase hydrolyzes only compounds in the L series and not in the D series (17, 18). A simple diagram (fig. 2) may be drawn to indicate this polyaffinity relationship for carboxypeptidase. The polar groups are bound through the metal, presumably magnesium, and the R groups are bound by the protein. From the polyaffinity concept and the knowledge that the metal is concerned in binding the polar groups, a beginning may be made in explaining the substrate specificities and inhibition properties of this enzyme. It is known that compounds which have free amino groups are either not hydrolyzed at all or are acted upon extremely slowly by this enzyme (12). It may be supposed that the metal has a greater affinity for the amino group than it does for the terminal carboxyl group, and a different type of complex is formed than in the usual enzyme-substrate complex. Such a combination with a free dipeptide is not a fruitful combination in that the substrate is not hydrolyzed. An explanation for the lack of action is that the linkages are not on opposite sides of the peptide bond but are on the same side (15).



In the same way we can explain why an L-amino acid like L-phenylalanine is not an inhibitor for carboxypeptidase; the combination given by an L-amino acid with the enzyme would be a weak one since no chelate rings are formed. However, Elkins-Kaufman and Neurath (19) found that D-amino acids are strong competitive inhibitors of this enzyme. We must suppose that the reason for this is that in the D-amino acid the R

group binding is extremely strong and that with this orientation a carboxyl group is not presented to the enzyme, but instead, a free amino group (15).

The validity of this concept can be partially tested by inhibition studies of various kinds. For example, the enzyme may be inhibited by blocking the metal portion of the enzyme or by blocking the R group position or both. Ideally, we should expect that the strongest inhibitors would be those that would bind both with the metal and with the R group position of the protein. From the type of scheme that has been indicated, we can predict the type of substances which would be expected to inhibit this enzyme. The sensitivity of the known substrates indicates that the strongest binding at the R position of the protein is given by those compounds that contain aromatic residues (12, 13, 17). It is also to be expected that those compounds that contain carboxyl groups would bind with the metal. It was found that benzylmalonic acid is such a compound. It has two carboxyl groups and it has a side chain much like that of phenylalanine, and it is indeed a strong inhibitor of carboxypeptidase (20). It was found that one mole of benzylmalonic acid combines with each active group of the enzyme. Interestingly enough, the unsubstituted dicarboxylic acid, malonic acid, is only an extremely poor and doubtful inhibitor of carboxypeptidase. Malonic acid at 0.5 M gives only slight inhibition in contrast to the 50 per cent inhibition produced by benzylmalonic acid at 0.003 M. Similarly, other compounds which can block the R group position and which bind with the metal also proved to be strong inhibitors. Such a compound as benzoic acid is a weak inhibitor but phenylacetic acid is quite a strong inhibitor. Likewise, indolacetic acid and indolpropionic acid have been found to be strong inhibitors of carboxypeptidase. From a series of inhibitors of different configuration, we can begin to evaluate and understand the precise limits of the configurations required for binding with the R group and with the metal simultaneously. We can measure the distances in terms of the number of carbon atoms between the functional groups, we can vary the substituents on the aromatic ring, and we can vary the kinds of functional groups. Such studies are now in progress.

What further can be done with these ideas of metal binding with the substrate? As we have indicated the normal enzyme-substrate combination occurs through chelate ring formation of the metal and the polar groups of the substrate. We must assume, as is known from many studies of inorganic and organic compounds, that the chelation is extremely strong indeed and that where ring formation is not possible, only weak combination occurs. How strong the chelation is between metals and amino acids or peptides is beautifully illustrated by an observation made by Neuberg and Mandl (21). They found that the metal ions known to be important in biological systems could not be precipitated as metal sulfides when amino acids or peptides were present. Moreover, they found that when an amino acid or peptide was added to an insoluble sulfide like zinc sulfide, the sulfide went back into solution. This indicates that the combination of the metal with amino acids or peptides is extremely strong indeed, and such a strong combination must be what is occurring in the enzyme-substrate complexes of the peptidases. The combination in the enzyme suggests that a strong electronic pull is exerted by the metal through the polar groups of the substrate to produce an electronic distortion or deformation at the sensitive peptide linkage (15). This deformation is presumably made still stronger through the combination of the metal with the protein; in other words, we must assume that the rôle of the protein, or part of the rôle of the protein in enzymes, is to make the metals even more electropositive than they normally are. The strong electropositive character increases the deformation at the peptide bond and that is what we mean, then, by the lowering of the free energy of the activation. In other words, the rôle of the enzyme as a catalyst is to lower the free energy of activation, and we can assume that this is caused in some way by the electronic pull through the metal. If this hypothesis is true, the real hydrolysis of the peptide bond is probably due to the acidic or basic catalysis of the hydrogen and hydroxyl ions of the aqueous solution. Hydrogen and hydroxyl ions can catalyze the hydrolysis of the peptide bonds. Normally, this hydrolysis occurs only in strongly acid or strongly alkaline solutions, but with a lower free energy of activation through the mediation of the enzyme, this catalysis now occurs in neutral solution.

As a consequence, the enzymatic hydrolysis of the peptides should follow many of the relationships which govern acid and base catalysis. For example, it is well known that with a series of related esters or amides the rate of the hydrolysis is a function of the strength of the acid which is

linked in the ester or amide bond (22). Examination of known substrates for carboxypeptidase shows this relationship to hold. For example, chloroacetylphenylalanine is hydrolyzed very much more rapidly than acetylphenylalanine. Obviously, chloroacetic acid is a far stronger acid than acetic acid. Similarly, it was reported last year (23) that carbobenzoxy-β-alanylphenylalanine is hydrolyzed about a thousand times more slowly than carbenzoxyglycylphenylalanine. Again it has been found that carbobenzoxy-β-alanine is a much weaker acid than carbobenzoxyglycine (20). In other words, a substantial portion

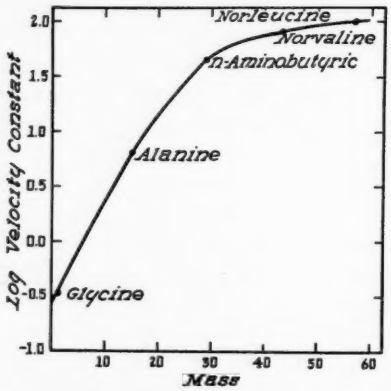


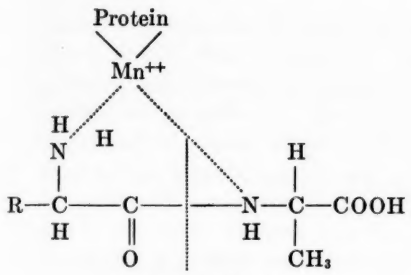
Fig. 3. ACTION OF LEUCINE AMINOPEPTIDASE ON various aliphatic amino acid amides. The logarithm of the relative first order velocity constant is plotted as a function of the mass (molecular weight) of the side chain bound to the alpha carbon. The initial slope indicates that the rate of hydrolysis is directly proportional to the mass (24).

of what has been called the specificity of an enzyme is not specificity at all but is a function of the free energy of the bond which is exposed to the action of the enzyme.

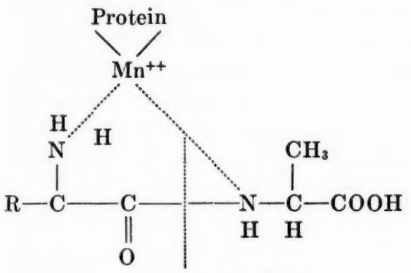
Thus, a beginning may be made in evaluating a number of distinct factors concerned in the specificity of these enzymes. First of all, as already indicated, there is the di-affinity concept of Euler and Josephson. We interpret this as the ability of the metal to form a chelate complex with the substrate. Secondly, there is the poly-affinity concept of Bergmann and his collaborators. The R groups must lie in a definite spatial position in relation to the polar groups. This is a second factor in specificity. A third factor, which was mentioned just a moment ago, is the free energy of the sensitive bond in terms of the acid strength of the carboxyl group which is linked in the peptide bond. Correspondingly, the energetic contribution of the amino group, imino group or ester group will also play a rôle in determining the rate of hydrolysis of such substrates.

A fourth factor may be termed the true specificity as determined by the protein; namely, the binding of the R group by the protein itself and the contribution made by this binding in terms of lowering the free energy of activation. The R group specificity has been measured very carefully with leucine aminopeptidase (24). Here we find, in contrast to former assumptions that this peptidase attacks only leucine compounds, that this enzyme hydrolyzes a variety of peptides containing different aliphatic residues. The simplest compounds are the homologous aliphatic amino acid amides and strictly comparable data have been obtained for their hydrolysis. It appears from these data (fig. 3) that the binding by the protein of the side chain is a function of the mass of the side chain in terms of its molecular weight. This suggests that the binding forces involved may perhaps be those of Van der Waal's forces since it is difficult to visualize other forces which are capable of binding aliphatic side chains. Here is a fourth effect which determines the specificity of these enzymes.

A fifth factor is one which appears to be a steric effect. With leucine aminopeptidase we find that L-leucinamide is hydrolyzed very rapidly; on the other hand, D-leucinamide is hydrolyzed at less than one-ten thousandth of the rate (7, 24). We cannot fix this in absolute terms since it is almost impossible to make absolute kinetic measurements involving such a slow hydrolysis. The difference in rates between these L and D compounds is obviously due to the polyaffinity relationship; namely, the isobutyl side chain of the D-amino acid amide lies in such a position that it cannot combine with the protein at the same time as there is a combination of the amino group and the peptide nitrogen with the metal. When the relative rate of hydrolysis of L-leucyl-L-alanine is compared to L-leucyl-D-alanine a different optical effect is observed. Here the L-compound is hydrolyzed about 25 times more rapidly than its diastereoisomer, L-leucyl-D-alanine (24). Since the residue attached to the nitrogen of the sensitive peptide bond does not appear to have any affinity for the protein, this must be interpreted as a steric effect in which the methyl group of the D-alanine lies in such a position as to interfere with the combination of the metal with the peptide nitrogen.



L-Leucyl-L-Alanine



L-Leucyl-D-Alanine

The residue attached to the peptide nitrogen does not seem to be bound by the protein because the rates of hydrolysis for a variety of compounds are almost identical; L-leucinamide, L-leucylglycine, L-leucyl-L-alanine, and L-leucyl-β-alanine are all hydrolyzed at the same rate so that the presence or absence of a carboxyl group or the change in position from an α-amino group to a β-amino group has no effect whatsoever. Thus, there is a fifth effect which is steric and which can influence the specificity of these enzymes.

To summarize, it may be remarked that while most earlier concepts of enzyme specificity were represented almost exclusively in terms of vague concepts such as lock and key mechanisms, we can now utilize the available information to evaluate separately at least five factors which determine the specificity of an enzyme. Undoubtedly, many other factors will also be discovered. The peptidases represent an extremely favorable group of enzymes in which to investigate these factors because the genius of Emil Fischer and Max Bergmann has given us the techniques for varying the potential substrates almost at will.

Our concepts of how enzymes work has been presented for the peptidases because we seem to be in a better position to do so with this group of enzymes. Obviously, two questions emerge in terms of the general theory which has been presented. First of all, do these concepts apply to other metal enzymes? Perhaps the simplest illustration as to how they apply may be shown with carbonic anhydrase (15). Here is an enzyme which contains zinc as the active metal as shown by the work of Keilin and Mann (25). The substrate, bicarbonate ion, is extremely simple in character and there can be no great concern about R group binding with the protein. In fact, this factor may be neglected completely and we may suppose that zinc forms a chelate complex with the protein on one hand and with two of the oxygens of bicarbonate ion on the other. Since the bicarbonate ion has a resonating structure it doesn't matter which two are postulated. Hydrolysis is obviously a basic catalysis. After the splitting occurs, carbon dioxide is bound to one valence of the zinc and hydroxyl ion with the other. Since this enzyme is reversible in its action, these are the bindings that lead to the formation of bicarbonate ion. It should be emphasized that the types of linkages that have been postulated are not hypothetical. Zinc binds strongly with carbonate and zinc carbonate is an extremely insoluble substance with little tendency to dissociate. Likewise at *p*H 7.0 it is well-known that zinc forms subcarbonates which are mixed compounds containing both hydroxyl ions and carbonic acid. Thus, the types of linkages that have been assumed for the reversible action of carbonic anhydrase are identical with those known to exist for ordinary inorganic combinations of zinc.

Similarly, the β-keto-carboxylic acids like oxalosuccinic acid and oxaloacetic acid form beautiful spectroscopic chelate complexes with metal ions as reported by Kornberg, Ochoa and Mehler (26). In fact, with some of these compounds the combination with the metal is sufficient to lower the free energy of activation enough so that decarboxylation proceeds. With others, the protein is required in order to make the metal more electropositive and to lower the free energy of activation still further. Oxalosuccinic acid is rather unstable at *p*H 7.0 in the absence of any outside agents; adding a trace of metal which forms a chelate complex lowers the free energy of activation enough to allow the decarboxylation to proceed. On the other hand, acetoacetic acid is rather stable in aqueous solution and combination with metal alone does not lower the free energy of activation sufficiently. Here the specific protein is required. In a similar manner, these concepts of the chelate complexing of metals may be applied to various other metal enzymes such as the phosphatases, phosphorylases, etc.

A second question is, what about those enzymes

which do not contain metal ions. The discovery of the metal in carboxypeptidase represents an object lesson in our type of thinking. Perhaps other enzymes which are known to exist in fairly homogeneous form should be tested for the presence of metal ions. On the other hand, there are many enzymes like crystalline trypsin, which do not contain metal ions. We examined the action of various substances on crystalline trypsin and could not find any inhibition whatsoever by the usual metal poisons (11). Here our thinking must be different.

Since it is probable that all enzymes function by a fundamentally similar mechanism, let us assume that the chelate character of the enzyme-substrate combination is correct, and that the lowering of the free energy of activation is due in each instance to electronic deformation. Then, where there is no metal present, the rôle of the metal ion must be taken over by other groups within the protein itself. There are extremely electropositive and electronegative groups in proteins. It would be useless at the present time to speculate as to the nature of these active groupings in the various enzymes. This is an experimental task for the future. However, it is well to recall at this point the extremely valuable and fundamental observations of Bergmann, Fruton and their collaborators (1) on the synthetic substrates of trypsin and other proteinases. Trypsin hydrolyzes synthetic peptide derivatives which contain free polar groupings; benzoylargininamide contains a free guanido group, benzoyllysinamide contains a free epsilon amino group. We must suppose that there is a combination between these polar groupings and groups at the peptide linkages with as yet unknown groups in the enzymes.

REFERENCES

1. Bergmann, M. and J. S. Fruton in F. F. Nord and C. H. Werkman. *Advances in Enzymol.* 1: 63, 1941.
2. Michaelis, L. and M. L. Menten. *Biochem. Ztschr.* 49: 333, 1913.
3. Chance, B. *J. Biol. Chem.* 151: 553, 1943; *Acta Chem. Scandinav.* 1: 236, 1947.
4. Keilin, D. and T. Mann. *Proc. Roy. Soc. London, s. B.* 122: 119, 1937.
5. Von Euler, H. and K. Josephson. *Ztschr. f. physiol. Chem.* 157: 122, 1926.
6. Johnson, M. J. and J. Berger, in F. F. Nord, and C. H. Werkman. *Advances in Enzymol.* 2: 69, 1942.
7. Smith, E. L. and M. Bergmann. *J. Biol. Chem.* 138: 789, 1941. *Ibid.* 153: 627, 1944.
8. Smith, E. L. *J. Biol. Chem.* 163: 15, 1946.
9. Smith, E. L. *J. Biol. Chem.* 173: 571, 1948. *Ibid.* 176: 21, 1948.
10. Anson, M. L. *J. Gen. Physiol.* 20: 663, 1937.
11. Smith, E. L. and H. T. Hanson. *J. Biol. Chem.* 176: 997, 1948. *Ibid.* in press.
12. Hofmann, K. and M. Bergmann. *J. Biol. Chem.* 134: 225, 1940.
13. Smith, E. L. *J. Biol. Chem.* 175: 39, 1948.
14. Snoke, J. E., G. W. Schwert, and H. Neurath. *J. Biol. Chem.* 175: 7, 1948.
15. Smith, E. L. *Proc. Nat. Acad. Sc.* 35: 80, 1949.
16. Bergmann, M. *Harvey Lectures*, 31: 37, 1935–36.
17. Stahmann, M. A., J. S. Fruton, and M. Bergmann. *J. Biol. Chem.* 164: 753, 1946.
18. Hanson, H. T. and E. L. Smith. *J. Biol. Chem.* In press.
19. Elkins-Kaufman, E. and H. Neurath. *J. Biol. Chem.* 175: 893, 1948.
20. Lumry, R. W., W. J. Polglase, and E. L. Smith. Unpublished observations.
21. Neuberg, C. and I. Mandl. *Arch. Biochem.* 19: 149, 1948.
22. Hammett, L. P. *Physical Organic Chemistry.* New York: McGraw-Hill, 1940.
23. Hanson, H. T. and E. L. Smith. *J. Biol. Chem.* 175: 833, 1948.
24. Smith, E. L. and W. J. Polglase. *Federation Proc.* 8: 252, 1949.
25. Keilin, D. and T. Mann. *J. Biochem.* 34: 1163, 1940.
26. Kornberg, A., S. Ochoa, and A. H. Mehler. *J. Biol. Chem.* 174: 159, 1948.

UPTAKE OF LABELED AMINO ACIDS BY TISSUE PROTEINS IN VITRO[1]

HENRY BORSOOK, CLARA L. DEASY, ARIE J. HAAGEN-SMIT,
GEOFFREY KEIGHLEY AND PETER H. LOWY

From the Kerckhoff Laboratories of Biology, California Institute of Technology

PASADENA, CALIFORNIA

THE uptake of labeled amino acids by tissue proteins has been construed by workers in this field to be the same as protein synthesis. We shall return to this topic later. Assuming, for the moment, this to be true, there can be no question that those who have entered the field were influenced by the work of Schoenheimer and his colleagues (1) who proved that the rate of protein degradation and resynthesis *in vivo* was much faster than was envisaged in the theory of endogenous and exogenous protein metabolism. We, in the Pasadena laboratory, had come to this conclusion some years earlier (2). Our evidence was mainly indirect; some additional evidence came from crude labeling experiments, and some was inferred from data on the free energy of formation of the peptide bond. We referred to the dynamic steady state of breakdown and synthesis of protein as the 'continuing metabolism' of nitrogen. The response to this view, generally, was unsympathetic. The direct and incontrovertible evidence came from the experiments of Schoenheimer and his colleagues on the feeding of N^{15} labeled amino acids and ammonia. This work and the clearing up of the problems of creatine (3–5) and of creatinine (6–8) formation cut away all the support of the theory of endogenous and exogenous metabolism of protein.

We were emboldened then to look with labeled amino acids for evidence of protein synthesis *in vitro*.

In vitro studies employing tracers appear to offer the best chance, at present, of gaining some insight into the mechanism of the biological synthesis of protein. The following considerations indicate the experimental problem of finding evidence of protein synthesis *in vitro*. The minimum duration of the *in vivo* experiments with N^{15} labeled amino acids was 3 days; *in vitro* the maximum time is about 6 hours. On the basis that the rates *in vitro* would be the same as *in vivo* the concentrations of labeled amino acids likely to be in a tissue protein were, therefore, in the *in vitro* experiments 1/12 or roughly 10 per cent of those *in vivo*. Furthermore, whereas the whole organs or carcasses of several animals are available for analysis after an *in vivo* experiment, only 100 mg. of protein or less may be available after *in vitro* experiments, especially where one wishes to make a series of comparisons. Roughly, then, the amount of a labeled amino acid to be measured after an *in vitro* experiment is about 1 per cent or less of that after an *in vivo* experiment. With radioactive isotopes such as C^{14} and S^{35} this can be done easily. One tenth of a microgram of a labeled amino acid with a specific activity of 100,000 count/min/mg. can be measured. We have been able to use leucine and lysine with specific activities as low as 6000 counts/min/mg.

C^{14} is the isotope which has been used most in *in vitro* work so far; it is especially convenient because of its long half life of 5100 years and ease of measurement. S^{35} has a half life of 87 days. Tritium has been made available recently; its half life is 31 years.

Table 1 gives some figures on the amounts of different labeled amino acids taken up *in vitro* by intact cells of different animal tissues. Animal tissue slices or cells were suspended in a saline solution containing the labeled additive and incubated at 36 to 38°. The experimental time varied from 2 to 6 hours; the figures given represent approximately the maximum uptake and indicate the orders of magnitude in the longest time it is useful to run experiments of this kind.

The table shows that both indispensable and dispensable amino acids are taken *in vitro* by tissue proteins. Every tissue so far tested has

[1] The experimental data of the authors presented here were obtained in a project under the joint sponsorship of the United States Atomic Energy Commission and the Office of Naval Research.

taken up every labeled amino acid presented to it.

Of the normal tissues so far investigated the uptake was fastest in bone marrow cells. The rates were slower and approximately equal in liver slices and in diaphragm. It is interesting that the CO_2 of bicarbonate is so rapidly taken up into the dicarboxylic acids of proteins. The rapid uptake of labeled amino acids by bone marrow cells and diaphragm indicate that these tissues may be useful biological objects for a variety of physiological and pathological studies.

The figures on the amounts of labeled amino acids taken up have, as they stand, only an operational significance. From figures such as these one can calculate how many counts one is likely to find in the protein. A more relevant presentation of the results is as percentage of the labeled amino acids in the protein which had become labeled, and to compare those figures with comparable ones obtained by Schoenheimer and his colleagues with N^{15} labeled amino acids *in vivo*. For example, the latter workers found that after feeding N^{15} labeled leucine to a rat for 3 days 8 per cent of the leucine in the liver proteins was labeled (1); we may say, therefore, that in 4 hours about 0.5 per cent was labeled. Our figure for bone marrow cell proteins *in vitro* in 4 hours is 1 per cent. In rat liver slices Melchior and Tarver found 0.3 per cent of the methionine labeled in 4 hours; Zamecnik *et al.* found 0.23 per cent of the alanine labeled in 4 hours and 5 times this amount in hepatoma cells. The uptake of L-lysine by liver homogenate is about as fast as *in vivo* (14). These estimates, rough as they are, are sufficient to indicate that the uptake of labeled amino acids by tissue proteins is of the same order of magnitude *in vitro* as *in vivo*.

The uptake of labeled amino acids is much faster in embryonic and malignant than in normal adult tissues (table 2). Rabbit bone marrow cells which have the fastest uptake of any tissue in normal animals so far tested belong, of course, with embryonic tissues.

Mechanical damage to cells reduces the rate at which their proteins take up labeled amino acids

TABLE 1. UPTAKE OF LABELED AMINO ACIDS BY INTACT CELLS IN VITRO

REFERENCE	LABELED ADDITIVE	AMT. OF LABELED AMINO ACID/GM. PROTEIN
	Rat liver slices	
(9)	$NaHCO_3$	0.76 mg. Aspartic + glutamic
(10)	DL-Alanine	0.15 mg. Alanine
(11)	DL-Methionine	0.0069 mg. Methionine + 0.0028 mg. Cysteine
	Rat Diaphragm	
(12)	L-Leucine	0.28 mg. Leucine
(12)	Glycine	0.15 mg. Glycine
(12)	L-Lysine	0.054 mg. Lysine
	Rabbit Bone Marrow Cells	
(12)	L-Leucine	1.15 mg. Leucine
(12)	L-Lysine	0.67 mg. Lysine
(12)	Glycine	0.56 mg. Glycine
	E Coli, Non-proliferating	
(13)	DL-Methionine	0.58 mg Methionine

TABLE 2. UPTAKE OF AMINO ACIDS BY NORMAL, ADULT AND OTHER TISSUES

REFERENCE	LABELED ADDITIVE	AGE	COUNTS/MIN/MG. PROTEIN
	Rat Liver Homogenate		
(15)	Glycine	Fetal	375
		Adult	11
		TYPE	AMT OF LABELED AMINO ACID/GM. PROTEIN
	Rat Liver Slices		
(10)	DL-Alanine	Hepatoma	1.12 mg. alanine
		Fetal	0.88 mg. alanine
		Normal	0.15 mg. alanine

TABLE 3. UPTAKE OF AMINO ACIDS BY INTACT AND BROKEN CELLS

REFERENCE	LABELED ADDITIVE	HOW BROKEN	RATIO: UPTAKE IN INTACT CELLS / UPTAKE IN BROKEN CELLS
		Rat Liver	
(11)	DL-Methionine	Homogenized	4 to 41
(16)	Glycine	Homogenized	3
		Rat Diaphragm	
(12)	Glycine	Homogenized	4
(12)	L-Leucine	Homogenized	4
(12)	L-Lysine	Homogenized	5
		Rabbit Bone Marrow	
(12)	Glycine	Cytolyzed with water	No uptake in broken cells
(12)	L-Leucine	Cytolyzed with water	No uptake in broken cells
(12)	L-Lysine	Cytolyzed with water	No uptake in broken cells
(12)	Glycine	Lyophilized	No uptake in broken cells
(12)	L-Leucine	Lyophilized	No uptake in broken cells
(12)	L-Lysine	Lyophilized	No uptake in broken cells
(12)	Glycine	Frozen and thawed 6 times	No uptake in broken cells
(12)	L-Leucine	Frozen and thawed 6 times	No uptake in broken cells
(12)	Glycine	Cytolyzed with ether	Almost no uptake
(12)	L-Leucine	Cytolyzed with ether	Almost no uptake

(tables 3 and 4). The important point is that liver homogenate (table 5) does take up glycine, leucine and lysine at a considerable rate. The rate of uptake of glycine by an homogenate is, to be sure, slower than in liver slices, but only one third slower; and the rate of uptake of lysine is as fast as *in vivo*. The uptake of lysine by liver homogenate appears to be a special case of which we shall have more to say later.

But an homogenate is neither an enzyme solution nor a suspension of insoluble protein. A large fraction of the suspension consists of organized particles such as nuclei and mitochondria. When working with homogenates we are not free of organized structures; and the level of organization of the particles is much higher than even that of proteins.

Tissues differ in the rates at which they take up different amino acids (table 6). The differences in the absolute rates are much greater than that of the relative rates. Thus bone marrow cells take up the 3 amino acids five times as fast as diaphragm although the order is the same.

In the one case tested so far, that of lysine, the L-isomer is taken up by liver homogenate protein much faster than the unnatural D-form (table 7). A part *certainly*, and possibly *all*, of the effect with the D-lysine may be ascribed to the small amount of the L-form in the D-preparation used.

In the course of an experimental run the rate of uptake of labeled amino acids by rabbit marrow cells and rat diaphragm is nearly linear up to 3 to 5 hours (fig. 1). In the case of glycine (fig. 2) with liver homogenate the rate is logarithmic up to 6 hours (when uptake is plotted against the logarithm of the time a straight line is obtained). In all of these cases whether the rate is linear or logarithmic it does not decline appreciably up to 3 to 4 hours. *Lysine* in *liver* homogenate again is an exception; the reaction comes to a stop in 2 hours (fig. 2).

The dependence of the rate of uptake on the initial concentration of labeled amino acid is in all cases but one up to a concentration of 50 mg. per cent, (approximately 0.005 molal) logarithmic (figs. 3, 4 and 5). High concentrations may be somewhat inhibitory, as has been observed in other enzymatic reactions with amino acids. The exception again is the uptake of lysine by liver homogenate (fig. 5). The relation of uptake to initial concentration of labeled lysine is in contra-

TABLE 4. UPTAKE OF AMINO ACIDS BY LIVER HOMOGENATES: EFFECT OF VARIOUS TREATMENTS

REFERENCE	LABELED ADDITIVE	TREATMENT	RELATIVE ACTIVITY OF PROTEIN	
		Rat		
(16)	Glycine	None	100	
		Homogenized with distilled water	19	
		Frozen and thawed 6 times prior to being homogenized	6	
		Guinea Pig		
			WHOLE HOMOGENATE pH 6.2	SEDIMENTED FRACTION pH 7.5
(12)	L-Lysine	None	100	100
		Ether	78	72
		Frozen and thawed 6 times	110	90

TABLE 5. UPTAKE OF AMINO ACIDS BY WHOLE LIVER HOMOGENATE AND SEDIMENTED FRACTION

REFERENCE	LABELED ADDITIVE	LIVER FRACTION	AMT. OF AMINO ACID*/GM. PROTEIN
		Rat	
(16)	Glycine	Whole	.015 mg. Serine + glycine
	Glycine	Sedimented at 2500 *g*	.025 mg. Serine + .004 mg. Glycine
		Guinea Pig	
(14)	L-Lysine	Whole	0.13 mg. Lysine
	L-Lysine	Sedimented at 2500 *g*	0.38 mg. Lysine
(12)	Glycine	Sedimented at 2500 *g*	0.13 mg. Glycine + serine
	L-Leucine	Sedimented at 2500 *g*	0.11 mg. Leucine

TABLE 6. RELATIVE UPTAKE OF GLYCINE, L-LEUCINE, AND L-LYSINE BY DIFFERENT TISSUES

RABBIT BONE MARROW CELLS			RAT DIAPHRAGM			GUINEA PIG LIVER, SEDIMENTED FRACTION		
(12)	L-Leucine	100	(12)	L-Leucine	100	(12)	L-Lysine	100
	L-Lysine	73		L-Lysine	79		Glycine	34
	Glycine	51		Glycine	34		L-Leucine	29

TABLE 7. UPTAKE OF L-LYSINE AND D-LYSINE BY GUINEA PIG LIVER HOMOGENATE

REFERENCE	LABELED ADDITIVE	ACTIVITY OF PROTEIN: COUNTS/MIN/MG. PROTEIN
(14)	L-Lysine	2.5
	D-Lysine[1]	0.6

[1] Contained 7–15% L-lysine.

distinction to all the other cases linear instead of logarithmic.

The important point in the data of figures 3, 4 and 5 is that the concentrations of labeled amino acids from which tissues can incorporate them into their proteins are of the order of those in the blood. And the dependence of the rate of uptake on the concentration of labeled amino acid is greatest in the physiological range of concentration.

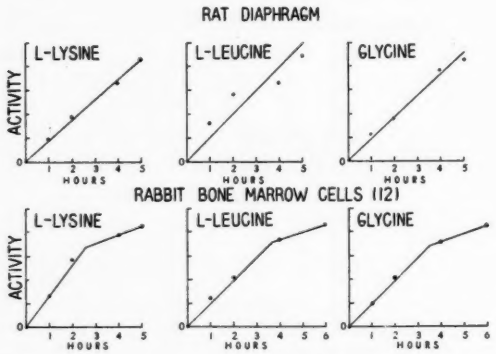


FIG. 1. RATES of uptake of amino acids (12)

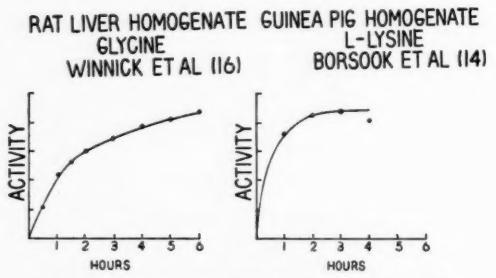


FIG. 2. RATES of uptake of amino acids

Some years ago we determined the free energy of formation of the peptide bond in a number of simple di- and tri-peptides. It was clear that over the *p*H range from 4 to 8 and at any practical amino acid concentration the equilibrium position was beyond 99.9 per cent hydrolysis of the peptide. Ionization constants and solubilities were taken into account. Peptide synthesis, under physiological conditions, could not, therefore, be a simple mass action reversal of hydrolysis (2). The process must be coupled with an energy donating reaction. Most of the protein in the cell is unhydrolyzed; and not because the system is inert; Schoenheimer and his colleaguess proved that the proteins of all tissues are continually being broken down and resynthesized. At thermodynamic equilibrium the protein of the body would be practically all hydrolyzed if not coupled with energy donating reactions. The steady state with regard to proteins is maintained where it is by the respiration of the cell.

It was to be expected, therefore, that if the uptake of the labeled amino acids by tissue proteins

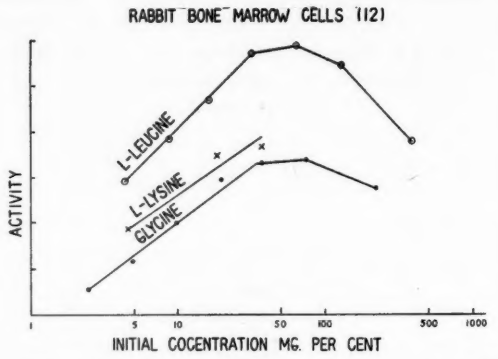


FIG. 3. EFFECT ON UPTAKE of concentration of amino acids. The abscissa is on a logarithmic scale; ordinates for each curve are fractions of the maximum.

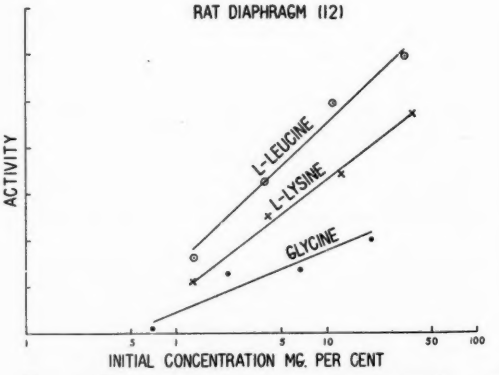


FIG. 4. EFFECT ON UPTAKE of concentration of amino acids. The abscissa is on a logarithmic scale; ordinates for each curve are fractions of the maximum.

is, indeed, peptide synthesis that this process would be inhibited by inhibition of respiration. Figure 6 shows that this is the case. The effects of dinitrophenol and of arsenate suggest that respiration promotes peptide synthesis by way of phosphorylation.

The foregoing observations are in accord with those of all other workers. Thus Melchior *et al.* (13) found that the uptake of methionine by *E. coli* was inhibited by azide, fluoride and cyanide; Winick *et al.* (16) found that oxygen consumption and glycine uptake by rat liver homogenate went together and that the process was inhibited by

anaerobiosis. Frantz *et al.* found that anaerobiosis (17) and dinitrophenol (18) inhibited the uptake of alanine by rat liver slices.

The exception, again, is the uptake of lysine by the proteins of guinea pig liver homogenate. Table 8 summarizes some of the major differences in the process of lysine uptake by liver and that of all other amino acids in all tissues so far examined. Lysine is taken up by at least two different processes in liver homogente, characterized by differences in *p*H optimum, dependence on calcium ions, sensitivity to fluoride and to oxidation and phosphorylation inhibitors (14).

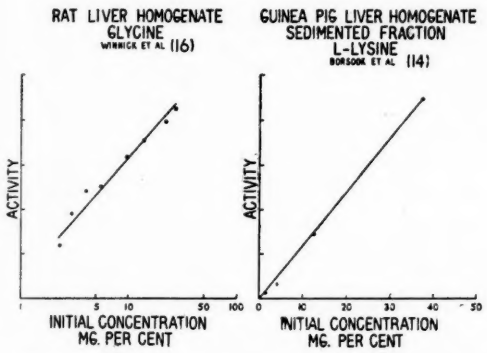


FIG. 5. EFFECT ON UPTAKE of concentration of amino acids. The abscissae for the glycine and lysine curves are logarithmic and linear, respectively; ordinates are fractions of the maximum.

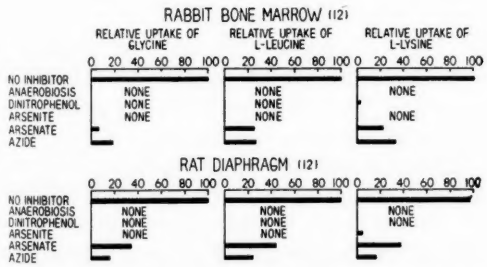


FIG. 6. UPTAKE OF AMINO ACIDS in presence of oxidation and phosphorylation inhibitors. The concentration of the inhibitors was 0.001M.

The findings with lysine caution against generalization even with respect to so elementary a condition as dependence of amino acid uptake on respiration. Synthesis of peptides by coupled reactions between peptides and hydrolytic enzymes, as discovered by Bergmann and his collaborators (19), may play a part; and these would not depend on respiration. Or syntheses analogous to those of disaccharides by sucrose phosphorylase discovered by Doudoroff *et al.* (20) may occur. In the latter case most of the free energy of the bond is retained in a complex of the enzyme and one cleavage product. Another cleavage product may then recombine with that on the enzyme and so reconstitute a peptide bond; and this process would be independent of respiration.

We have surveyed the effects of metal ions such as cobalt, copper and manganese on the one hand because of the well known effects of cobalt and of copper on hemoglobin and red cell formation, and on the other because the activity of a number of peptidases depends on these ions.

TABLE 8. COMPARISON OF CONDITIONS AFFECTING THE UPTAKE OF AMINO ACIDS IN DIFFERENT TISSUES (12).

	GLYCINE, LEUCINE AND LYSINE IN BONE MARROW CELLS AND DIAPHRAGM AND GLYCINE IN LIVER HOMOGENATE	LYSINE	
		Whole Homogenate	Sedimented Fraction
Optimum *p*H	7.3–7.5	6.2	7.5
Frozen and thawed	100% inhibition	No inhibition	No inhibition
Dependence on calcium	None	Obligatory	Slight
Anaerobiosis	100% inhibition	No inhibition	Slight inhibition
Arsenite	100% inhibition	50% inhibition	No inhibition
Arsenate	80–95% inhibition	24% inhibition	No inhibition
Azide	65–75% inhibition	20% inhibition	8% inhibition
Dinitrophenol	100% inhibition	28% inhibition	No inhibition
Dependence on concentration of amino acid	Logarithmic	Linear	Linear

Concentration of inhibitors was 0.001 M

In bone marrow cells cobalt, copper and manganese were inhibitory with all 3 amino acids over a range of .005 to .0001 molal. The final concentrations of the free metal ions in the reaction medium were certainly much less than these. Substantially the same effects were observed in diaphragm. Of the 3 metal ions copper was the most inhibitory and manganese the least. In liver, copper was inhibitory at an added concentration of 0.001M or higher. Manganese was not inhibitory and at concentrations below 0.001M there may have been a slightly increased rate of uptake. These findings argue against participation of peptidases in the uptake of labeled amino acids by tissue proteins.

The effects of cobalt in liver homogenate were especially interesting and point to the need of

caution in interpreting the effects of metal ions. Table 9 shows that the addition of cobalt ($Co\ Cl_2$) increased the counts which could not be washed out of the protein after the homogenate had been incubated with labeled leucine or glycine. But after the proteins (which had been exhaustively extracted with trichloroacetic acid) were treated with the ninhydrin reagent the CO_2 liberated gave a larger fraction of the counts originally in the protein. As the ninhydrin reagent liberates CO_2 from the carboxyl groups of amino acids only when both their carboxyl and amino groups are free, we have interpreted this result as indicating

TABLE 9. EFFECT OF COBALT ON UPTAKE OF L-LEUCINE AND GLYCINE BY GUINEA PIG LIVER HOMOGENATE: SEDIMENTED FRACTION (12)

CONCENTRATION OF $CoCl_2$ ADDED, MOLAL	COUNTS/MIN/MG. PROTEIN	AFTER NINHYDRIN TREATMENT OF PROTEIN: COUNTS/MIN/MG. PROTEIN		INHIBITION OF UPTAKE, %
		in CO_2 liberated	Calculated remaining in protein	
		L-Leucine		
0	3.0	0	3.0	
0.005	10.0	9.5	0.5	83
0.001	2.5	1.4	1.1	63
0.0005	2.6	0.7	1.9	36
0.0001	4.2	0.1	4.1	0
		Glycine		
0	7.4	0	7.4	
0.005	9.3	6.3	3.0	59
0.001	4.1	1.8	2.3	68
0.0005	7.7	1.3	6.4	13
0.0001	8.5	0.6	7.9	0

the formation of a protein-cobalt-amino acid complex. Similar results were obtained with lysine; but as the latter amino acid was labeled in the ϵ-position treatment with the ninhydrin reagent could not be used to demonstrate the protein-cobalt-amino acid complex. Table 9 shows that as far as the actual incorporation of amino acids into the proteins is concerned, cobalt was inhibitory, as it was in marrow cells and in diaphragm.

The degree of complex formation is greater the greater the concentration of added cobalt. Not all proteins form this complex with cobalt to the same degree. The most active we have found are those in the sedimented fraction of liver homogenate; diaphragm proteins come next; bone marrow proteins and egg albumin are the least active. Amino acids also vary in their ability to participate in this complex formation. Of the 3 amino acids we have tried the order of effectiveness is leucine, glycine and lysine. The formation of this complex takes about 6 hours to reach completion at 38°; it does not proceed below *p*H 6.0; and increases progressively at *p*H values above that.

Having several tissues each of which was capable of taking up glycine, leucine and lysine we were in a position to examine whether the amino acids mutually influence their uptake. The results summarized in figure 7 show that glycine, leucine and lysine are taken up independently in the 3 tissues tested. The counts in the protein when all 3 amino acids were added together was the sum of the 3 added separately.

In other experiments addition of 2 radio-inactive amino acids together with one which was radio-active did not affect the uptake of the latter. Nor did the addition of a mixture of amino

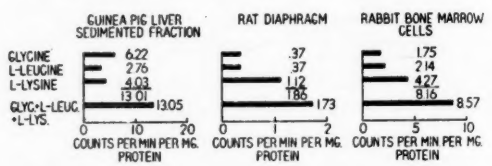


FIG. 7. UPTAKE OF AMINO ACIDS added separately and together (12).

acids approximating the composition of casein or of hemoglobin affect the uptake of labeled glycine, leucine or lysine.

This result needs to be correlated or reconciled with the observations in feeding experiments (21–25). It was found in those experiments that an indispensable amino acid is ineffective for growth or recovery from protein depletion unless it is fed within a few hours of other necessary amino acids. In all of the *in vitro* experiments so far the tissues no doubt contained all the normal amino acids to some extent.

A major assumption underlying all this work is that radioactivity in the protein after incubating a tissue with a radioactive amino acid means that the labeled amino acid has been incorporated into it by peptide bonds. The whole structure of interpretation rests on this assumption. It has not been proved.

After it was found possible to identify with the ninhydrin reagent the binding of labeled amino acids to protein by cobalt all the crucial experiments with bone marrow cells, diaphragm and liver homogenate with glycine and leucine were repeated and the ninhydrin test applied to their

radioactivity. The test is applicable to our glycine and leucine, which were labeled in their COOH groups, but not to the lysine, which was labeled in the ϵ-position. The CO_2 liberated by ninhydrin from all the radioactive proteins, in those experiments in which no cobalt was added, gave no counts. We may conclude, then, that the labeled amino acids were bound in such manner that either their COOH or their amino groups were masked, possibly by participation in peptide bonds but possibly also in bonds of some other kind.

In many of the experiments by other workers and in all of ours the labeled amino acid was recovered as such after acid hydrolysis of the protein. This showed that the radioactivity was carried into the protein by the labeled amino acid in such form that the amino acid could be recovered as such after complete acid hydrolysis.

The results of this test and of the ninhydrin test were necessary but they are not sufficient evidence; they are not proof that the labeled amino acids were incorporated by peptide bond formation.

It would be proof if a peptide could be isolated and identified among the partial hydrolysis products of a radioactive protein. To obtain such evidence bone marrow protein was obtained radioactive by incubating the cells with labeled leucine. An aliquot of the protein was treated with ninhydrin and the CO_2 evolved gave no counts. The protein was partially hydrolyzed with a mixture of strong acetic and hydrochloric acids for 10 days at 38°, and the hydrolysate was then chromatographed on a starch column according to the method of Moore and Stein (26). The effluent fractions were analyzed with the ninhydrin reagent for amino nitrogen and also for radioactivity. One region was found of coinciding ninhydrin color and of radioactivity. The position of this region was in advance of the position of leucine; in fact 2 non-radioactive ninhydrin bands intervened between it and the leucine band. Over 90 per cent of the radioactivity originally in the protein was in the one radioactive band.

Fractions at the peak of the radioactive band were pooled and completely hydrolyzed with HCl; and the hydrolysate was again chromatographed. The complete hydrolysate gave a starch chromatogram indicating 17 or more amino acids in the hydrolysate. All of the radioactivity was confined to the leucine plus isoleucine band. Carrier isolation was then applied to this radioactive fraction and all of the radioactivity was identified as being in the leucine. These results showed that partial hydrolyses of the radioactive protein liberated the incorporated radioactive leucine in a large peptide.

The objection to this particular piece of evidence is that the radioactive fraction in the partial hydrolysate emerged from the starch column too soon for good resolution; and the peptide fraction in which the radioactivity was found was too complex for determination of its structure.

We have repeated this experiment several times on bone marrow protein containing labeled glycine, leucine and lysine. Among the partial hydrolyzed products a large peptide was found containing leucine and lysine.

This kind of evidence brings us closer to rigorous proof that the labeled amino acids taken up by the protein are incorporated by means of peptide bonds; but it is not yet proof. It will be necessary to identify the peptides, isolate them and show that the labeled amino acids are constituent amino acids of the peptides.

TABLE 10. SYNERGISTIC ACTION OF FRACTIONS OF GUINEA PIG LIVER HOMOGENATE ON UPTAKE OF GLYCINE (12)

INCUBATED WITH FRACTIONS SEDIMENTED AT		RADIOACTIVITY OF PROTEIN	WEIGHT OF PROTEIN	TOTAL RADIOACTIVITY OF PROTEIN	
2500 *g*	30,000 *g*				
		Cts/min/mg.	*mg.*	*Cts/min.*	
+	−	0.59	60	35	43
−	+	0.14	59	8	
+	+	0.72	124	89	

However the evidence at hand, as far as it goes, is in accord with the interpretation that the labeled amino acids are taken up by tissue proteins in peptide bonds. There are no discrepancies and the evidence is quite varied in character.

Multiple labeling of protein followed by partial hydrolysis and isolation of the peptides promises to be a new and useful method of determining the detailed amino acid pattern of proteins.

Workers in the field of biological oxidations are finding that they have to deal with organized cytoplasmic particles. We have found this in the methylation of guanidoacetic acid by methionine in liver homogenate. We are now finding the same in this field. Table 10 summarizes a result we have obtained with labeled glycine in guinea pig liver homogenate. The fraction sedimented at 2500 *g* which consists largely of mitochondria, takes up glycine actively; the fraction sedimented

above 30,000 *g*, microsomes largely, take up much less; when the two fractions are incubated together the amount of glycine taken up is about double the sum of the two when incubated separately. There is clearly a synergistic action between the two sets of particles.

A summary of the situation in this field at present runs somewhat as follows. All the tissues so far tested can take up *in vitro* into their proteins every labeled amino acid which has been presented to them. The most active normal tissue in adult animals is bone marrow. The uptake of every amino acid in every tissue, with one exception, is immediately dependent on respiration. The exception is the uptake of lysine by liver homogenate. The effective range of labeled amino acid concentration is within the order of that in the plasma; and the rate of uptake is of the order of that *in vivo*. Glycine, leucine and lysine are taken up independently of each other. The evidence indicates that the amino acids are taken up in peptide bonds; but this point is not yet proved. There is evidence in the case of liver homogenate, at least, of a synergistic action between two sets of intracellular particles.

A one sentence summary is that the field of the biological synthesis of protein is now wide open.

REFERENCES

1. Schoenheimer, R. *The Dynamic State of Body Constituents.* Cambridge, Mass.: 1942.
2. Borsook, H., and G. L. Keighley. *Proc. Roy. Soc., London, s. B* 118: 488, 1935.
3. Borsook, H. and J. W. Dubnoff. *J. Biol. Chem.* 132: 559, 1940; 138: 389, 1941.
4. Bloch, K., and R. Schoenheimer. *J. Biol. Chem.* 138: 167, 1941.
5. Du Vigneaud, V., M. Cohn, J. P. Chandler, J. R. Schenck, and S. Simmonds. *J. Biol. Chem.* 140: 625, 1941.
6. Bloch, K., and R. Schoenheimer. *J. Biol. Chem.* 131: 111, 1939.
7. Bloch, K., R. Schoenheimer, and D. Rittenberg. *J. Biol. Chem.* 138: 155, 1941.
8. Borsook, H., and J. W. Dubnoff. *J. Biol. Chem.* 168: 493, 1947.
9. Anfinsen, C. B., A. Beloff, A. B. Hastings, and A. K. Solomon. *J. Biol. Chem.* 168: 771, 1947.
10. Zamecnik, P. C., I. D. Frantz, Jr., R. B. Loftfield, and M. L. Stephenson. *J. Biol. Chem.* 175: 299, 1948.
11. Melchior, J., and H. Tarver. *Arch. Biochem.* 12: 309, 1947.
12. Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. Lowy. Unpublished observations.
13. Melchior, J. B., M. Mellody, and I. M. Klotz. *J. Biol. Chem.* 174: 81, 1948.
14. Borsook, H., C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. Lowy, *J. Biol. Chem.* In press.
15. Friedberg, F., M. P. Schulman, and D. M. Greenberg. *J. Biol. Chem.* 173: 437, 1948.
16. Winnick, T., I. Moring-Claesson, and D. M. Greenberg. *J. Biol. Chem.* 175: 127, 1948.
17. Frantz, I. D. Jr., R. B. Loftfield, and W. W. Miller. *Science* 106: 544, 1947.
18. Frantz, I. D. Jr., P. C. Zamecnik, J. W. Reese, and M. L. Stephenson. *J. Biol. Chem.* 174: 773, 1948.
19. Bergmann, M., and J. Fruton. *Ann. New York Acad. Sc.* XLV: 409, 1944.
20. Doudoroff, M., H. A. Barker, and W. Z. Hassid. *J. Biol. Chem.* 168: 725, 1947.
21. Geiger, E. *J. Nutrition* 34: 97, 1947; 36: 811, 1948.
22. Cannon, P. R., C. H. Steffee, L. J. Frazier, D. A. Rowley, R. C. Stepto. *Federation Proc.* 6: 390, 1947.
23. Schaeffer, A. J., and E. Geiger. *Proc. Soc. Exper. Biol. & Med.* 66: 309, 1947.
24. Harte, R. A., J. J. Travers, and P. Sarich. *J. Nutrition* 35: 287, 1948.
25. Yeshoda, K. M., and M. Damodaran. *Biochem. J.* 41: 382, 1947.
26. Moore, S., and W. H. Stein. *J. Biol. Chem.* 176: 367, 1948; 178: 53, 1949.

MECHANISM OF PEPTIDE BOND FORMATION

FRITZ LIPMANN

From the Massachusetts General Hospital and the Harvard Medical School

BOSTON, MASSACHUSETTS

THE present understanding of the mechanism of peptide synthesis derives mainly from the study of relatively simple reactions. The animal body has fortunately supplied a number of model reactions for the study of synthesis of peptidic[1] links in two-component systems. Reactions of such a type are found among the so-called detoxication reaction; hippuric acid synthesis as well as acetylation of aromatic amine are relatively convenient model systems. The study of the acetylation of aromatic amines (1) in particular has relatively early furnished valuable detail which appears now rather generally applicable. It will, therefore, be advantageous to summarize first results obtained with this reaction.

ACETYLATION OF AROMATIC AMINES

Our interest in this reaction had originally been aroused by the finding of acetyl phosphate as an intermediary in a reaction chain geared apparently on generation of energy-rich phosphate bonds (2). The emergence of this particular molecule, however, suggested that it might not only be a link, as it indeed can be, in a phosphate transfer chain. It was suggestive to imply that acetyl phosphate may, with the appropriate catalyst, also offer its organic part in a state of activation for synthesis (3). This expectation lead rather early to the generalization that carboxyl activation by phosphorylation may be a major phase in biosynthesis (3).

After initial disappointments with the use of synthetic acetyl phosphate, it was observed that acetate plus adenyl pyrophosphate (ATP) would serve in pigeon liver extract as acetyl donor to aromatic amines (1). Using a protein fraction of pigeon liver extract a near equivalence of ATP breakdown and sulfonamide acetylation was obtained (4).

In table 1 a closely related reaction is included, namely, the activation of acetate by ATP to form hydroxamic acid in the presence of hydroxylamine. With a somewhat greater activity for hydroxamic acid formation in this extract, the stoichiometric relationship between acetylation and phosphate mineralization appears more clearly than in the case of sulfonamide acetylation. The overall condensation reaction is thus formulated:[2]

$$CH_3COOH + Ad\sim P\sim P + NH_2\cdot R \rightarrow CH_3\cdot CONH\cdot R + AD\sim P + P_{inorg.}$$

R stands here for either aromatic residue or the hydroxyl group of hydroxylamine.

The hydroxamic acid reaction has proven a very helpful tool in the investigation of acyl activation. Some time ago, quite accidentally, we had become aware of an explosive, non-enzymatic liberation of inorganic phosphate from acetyl— or other acyl—phosphate on addition of hydroxylamine. The corresponding hydroxamic acid is formed which gives with ferric ion a deeply purple colored complex. This reaction is used now for a colorimetric determination of the acyl phosphate (5). Furthermore, if added to an enzymatic system, hydroxylamine may act as a trap for activated carboxyl groups which thereby become accessible to determination by the color reaction (6, 9, 10). Due to the great reactivity of hydroxylamine, the evaluation of such results requires well controlled conditions (cf. 7).

The further establishment of the carboxyl group as the site of activation in peptidic synthesis appeared important. In view of the existance of an energy-rich N–P phosphate bond in creatine phosphate, the possibility of a primary phosphorylation of the amino group appeared plausible. As mentioned previously, experiments

[1] To simplify the following discussion, the term "peptidic link" is introduced as generic name for a —NH·CO-link between any amino or carboxyl group. The term "peptide link" is then reserved for the 1-carboxyl, 2-amino-link between two alpha amino acids as it occurs in protein.

[2] AD∼P∼P = ATP; Ad∼P = ADP; Ad = adenylic acid.

with synthetically prepared acetyl phosphate as acetyl donor for amines had proven unsuccessful. More recently, however, Dr. Kaplan studied in our laboratory a compound formed by enzymatic phosphorylation of acetate with ATP, or phosphopyruvate plus ADP, in extracts of *E. coli*. He found it to differ in some respects from synthetic acetyl phosphate. This 'natural' ATP-acetate reaction product appeared active as acetyl donor; the compound was shown to condense with formate in extracts of *E. coli* yielding pyruvate. It also acts, as shown in table 2, as acetyl donor for aromatic amines, significantly still requiring coenzyme A (8).

Table 1. Comparison of phosphate mineralization and amine acetylation or hydroxamic acid formation in fractions of pigeon liver extract[1]

Additions	$P_{inorg.}$	Acetyl sulfanilamide	Hydroxamic acid
	μM formed	*μM formed*	*μM formed*
	0	0	0
ATP	0.79		
" + sulfanilamide	1.24	0.64	
" + hydroxylamine	1.83		1.4

[1] All samples contain acetate and coenzyme A.

Table 2. Sulfanilamide acetylation with 'natural' acetyl phosphate[1]

Acetyl phosphate	ATP	Coenzyme A	Sulfanilamide acetylated
μM	*μM*		*μM*
8, synthetic	1	+	0.15
	10[2]	+	0.85
6, natural		+	0.78
6, natural			0.23

[1] 1 ml. pigeon liver extract incubated 2 hrs. at 25°.

[2] + acetate

This observation then allows us to divide the overall reaction into two steps,

$$\text{acetate} + \text{Ad}\sim\text{P}\sim\text{P} \rightarrow \text{acetyl}\sim\text{P} + \text{Ad}\sim\text{P} \quad (1)$$

$$\text{acetyl}\sim\text{P} + \text{amine} \rightarrow \text{acetyl-amine} + P_{inorganic} \quad (2)$$

The intermediate formation of an acyl phosphate in the synthesis of a peptidic link is therewith established. It remains, however, still doubtful if the two steps represent separable enzyme systems.[3]

GLUTAMINE

We now turn to the discussion of a very similar but more truly 'peptidic' reaction, the synthesis of glutamine. Speck found this reaction in acetone pigeon liver extract (9) prepared analogously to the one used in our sulfonamide acetylation experiments. In such an extract, glutamic acid, ATP and ammonia yield anaerobically glutamine when cysteine and magnesium ion are present. So far no indication for an involvement of coenzyme A in this reaction has been found. It is significant that hydroxylamine is practically as effective an acceptor as ammonia. This reaction is very rapid. In the experiment shown in table 3, 0.15 ml. extract were used only and in 15 minutes nearly 2 micromoles of glutamine were formed.

Table 3. Glutamine synthesis in pigeon liver extracts[1]

System	with 0.01 M NH_4Cl		with 0.3 M NH_2OH
	Amide formed	Inorganic phosphate formed	Hydroxamic acid formed
	μM	*μM*	*μM*
Complete.....	1.91	2.46	1.41
Without ATP.....	0.08		0.14
Without glutamate.	0.13	0.54	0.11

[1] Speck, J. F. *J. Biol. Chem.* 168: 402, 1947.

With this rapid synthesis and a relatively low background ATP-decomposition the stoichiometric relationship between ATP breakdown and glutamic acid-ammonia condensation appears most clearly. Such rapid phosphate transfer followed immediately by condensation leading eventually to phosphate liberation is remarkable. It should make one very cautious not to mistake an ATP-decomposition of such a type for ATP hydrolysis. For instance, with some ammonium ion present, as is the rule in any crude tissue extract, an ATP breakdown with added glutamate, due to glutamine formation, could easily be mistaken for a 'glutamate-catalyzed ATP-ase' effect.

Simultaneously with Speck, Elliott in Cambridge, England, found in acetone brain extract (10) and in bacterial preparations (11) an analogous glutamine and glutamhydroxamic acid syn-

[3] Recently a separation of the two enzymes, catalyzing steps 1 and 2, was accomplished by acetone fractionation of pigeon liver extract in the cold (14).

thesis (table 4). Although Speck as well as Elliott was not able to show an acyl phosphate accumulation in the absence of an acceptor, the reaction with hydroxylamine strongly indicates carboxyl activation as a primary reaction.

HIPPURIC ACID

A third peptidic synthesis of this type was found to be hippuric acid condensation. The p-aminohippuric acid synthesis has been studied extensively by Cohen and his group (12) in rabbit liver homogenate. In this case the synthetic activity is particle-bound. The figure 1 from Cohen's paper (12) shows anaerobic synthesis due to addition of ATP. Because of the considerable

TABLE 4. GLUTAM-HYDROXAMIC ACID IN BRAIN EXTRACTS[1]

SYSTEM	HYDROXAMATE[2]	PHOSPHATE[2]
Complete	2.3	1.9
No glutamate	0	0.1
No ATP	0	0
No Mg	0.3	0.2

[1] ELLIOTT, W. H. *Nature* 161: 128, 1948.
[2] Data for hydroxamate formation and phosphate liberation in μM/sample.

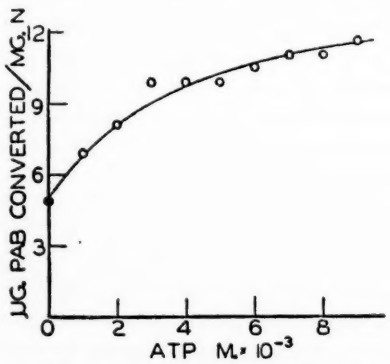


Fig. 1. SUPPORT of anaerobic hippuric acid formation by ATP. Rat liver homogenate in nitrogen (12).

background decomposition of ATP, a large excess of this reagent has to be applied in such a particle suspension, which obviates experiments on the stoichiometry.

These three examples of peptide bond formation summarized in figure 2, we may conclude, present a thoroughly analogous situation: the energy-rich phosphate of ATP acts as immediate energy source, attaching itself primarily to the carboxyl component. Certainly this is so in the case of acetylation and presumably also in glutamine and hippurate synthesis. The inability to isolate the primary reaction products in tissue experiments at least, may be due to the presence there of a very active acyl phosphatase in such preparations (13). It should be pointed out, however, that in Speck's experiments glutamic acid alone causes only little if any specific ATP breakdown. And, conversely, no ATP breakdown occurs with ammonia and hydroxylamine alone. This may be due to a special construction of the enzyme which permits reaction only if both the primary phosphate acceptor and the second participant in the condensation reaction are present. Or, the equilibrium conditions or the transfer from ATP allow no appreciable accumulation of the reaction product. The definite answer to such rather important questions of finer reaction mechanism may be obtainable by fractionation of pigeon liver extract and such experiments are in

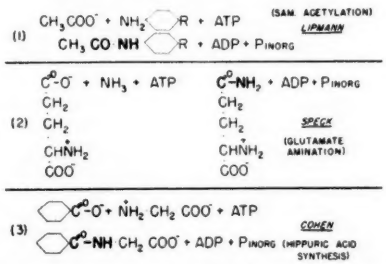


Fig. 2. SYNOPSIS of ATP-dependent peptidic syntheses. Two-component systems.

progress. Presently, we have only found that glutamine synthesis and acetylation may quite easily be separated on fractionation with ammonium sulfate (14).

GLUTATHIONE

So far we have considered only two-component systems and it is not too surprising that at this still exploratory stage best information on the mechanism is available with these relatively simple reactions. Promising progress, however, was made recently with the study of the mechanism of synthesis of a tripeptide, namely glutathione.

After the use of isotopes had shown a rapid turnover rate with this abundant cell constituent, Bloch reported to have obtained in liver homogenate support of anaerobic incorporation of radioactive glycine when ATP was added (15). No exchange occurred in the absence of a phosphate donor. He now has been able to obtain the reaction in acetone pigeon liver extract, which seems to be an almost inexhaustible source of ATP

linked reactions. These recent results were kindly made available to me by Dr. Bloch before publication (16) and are represented in table 5.

The glycine incorporation indicates here an ATP-driven synthesis of a true peptide link between the cysteine and glycine (cf. fig. 3). The rather rapid rate of incorporation suggests this system as a good starting point for the exploration of the synthesis of more complicated structures.

RELATION TO PROTEIN SYNTHESIS

It is of course uppermost in our minds how far the experience gained with few-component sys-

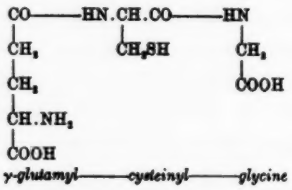


Fig. 3. FORMULA of glutathione

TABLE 5. INCORPORATION OF C^{14}-GLYCINE INTO GLUTATHIONE IN EXTRACTS OF ACETONE-DRIED PIGEON LIVER[1]

Each flask contained 5 ml. of centrifuged extract (0.5 gm. acetone pigeon liver), 1.2 ml. of 0.32 M glutamate, 1.2 ml. of 0.16 M C^{14}-glycine (relative specific activity 100,000 cts/min.), 0.4 ml. 0.16 M cysteine, 1 ml. 0.15 M KCl, 2.5 ml. of 0.1 M phosphate buffer (*p*H 7.4) and 0.5 ml. 0.15 M $MgSO_4$. Total volume, 12.2 ml. The solutions were incubated in N_2 at 37° for 1 hr.

ADDITION	RELATIVE SPECIFIC ACTIVITY IN GLYCINE MOIETY OF GLUTATHIONE *Cts. C^{14}/min.*
ATP 0.001 M	3315
None	73
ATP 0.001 M	2194
NH_4Cl 0.002 M	

[1] BLOCH, C. *J. Biol. Chem.* In press.

tems may be applicable to the understanding of the synthesis of a specific protein structure. The problem of the synthesis of protein, or for that matter any high molecular compound, may tentatively be divided into a polymerization- and a patternization-problem. A separate study of the polymerization of amino acids would probably be most easily performed on a polypeptide containing a single species of amino acids only. To my knowledge the only known representative of this type is the D-glutamic acid polypeptide produced by *B. anthrax* and similar organisms (17). The exploration of the mechanism of such synthesis may well be a worthwhile enterprise. A promising approach to the second, the patternization problem, seems already to open with the now possible study of glutathione synthesis.

Such questions of detailed mechanism have so far not been really touched although the general mechanism of protein synthesis was studied extensively by use of isotopes. With a number of amino acids (18, 19) at least the need of respiratory energy supply for incorporation of amino acids into protein has been established. A somewhat further breakdown of the therewith evidenced energy requirement has been obtained by inhibition studies using dinitrophenol. It was reported recently that dinitrophenol specifically interrupts the link between respiration and phosphate bond generation without affecting hydrogen transfer (20). This selective effect of dinitrophenol was utilized by Frantz, Zamecnik *et al.* (21) to test more specifically for the energy link to amino acid incorporation. They studied the effect of dinitrophenol on alanine incorporation into liver and tumor protein. Their results with normal liver slices are represented in the right hand curve of figure 4. Dinitrophenol, it apears, blocks amino acid incorporation without reduction or rather with an increase in respiratory oxygen consumption. A similar result was obtained with tumor slices. These data are in figure 4 compared with the effect of dinitrophenol on phosphorylation as observed with kidney homogenate, using yeast hexokinase plus fructose as the phosphate acceptor. Except for a slight difference in concentration, the parallel of the dinitrophenol effect on phosphorylation and protein synthesis, respectively, is striking and it suggests a phosphate bond transfer to be operative in the process of incorporation of amino acid into protein. This observation then seems to align the exhaustively studied reactions in two- or three-component systems with peptide formation towards the larger protein molecules. Such does not exclude, however, the fact that in the building of more specialized structures exchange reactions on the 3000-calories level (cf. fig. 5) may participate. This exchange could well be catalyzed by so-called hydrolytic enzymes, in a manner analogous to the exchange reactions recently described by Axelrod (22) with acid phosphatase.

ENERGY RELATIONS

Even without a complete knowledge of the finer mechanism of the condensations, it appears

clear that a single energy-rich phosphate bond is used to effect a single peptide link. This is evidenced by the one to one relationship between synthesis and phosphate liberation. Judging plainly from the energy data, the possibility would exist that an energy-rich phosphate bond could cover two or even more peptide links. This, however, would anyway be unlikely for reasons of reaction mechanism.

The energy requirement for peptide synthesis has been determined by Borsook (23). His earlier data were recently revised and extended and Dr. Borsook has kindly permitted me to use his most recent and as yet unpublished results. This and related data are listed in table 6. The energy figures for a number of typical peptides show no great deviation from the average and fall inside the limits of 2500 and 3000 calories per peptide bond.

On the other hand, the pyrophosphate link in adenyl pyrophosphate represents 12,000 calories (3). This means that excess energy is spilling over with a net utilization of around 20 to 25 per cent in the formation of the new bond. The scheme of figure 5 indicates such correlation between energy donor and acceptor system to be a very general occurrence in cellular synthesis.

As has been earlier pointed out elsewhere (3), there is a distinct grouping around two energy levels, a higher one at 12,000 calories and a lower at 3000 calories. The energy-rich phosphate bond carries excess energy if performing condensation

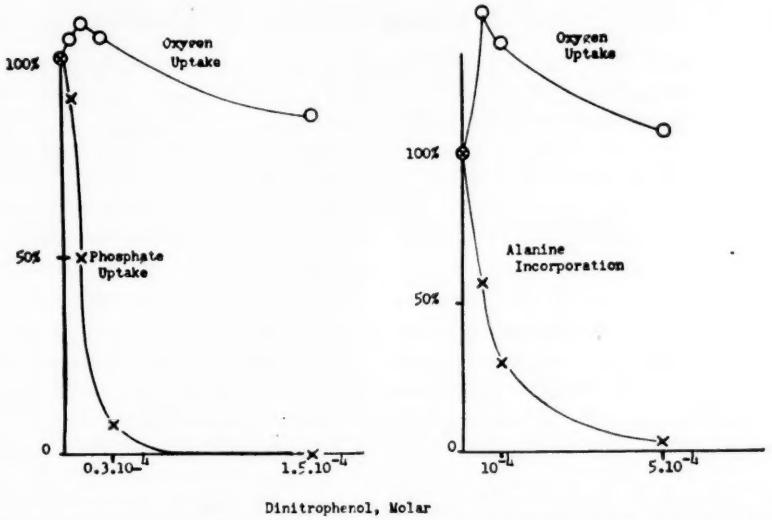


Fig. 4. *m*-DINITROPHENOL INHIBITION. Comparison of the inhibition by dinitrophenol of phosphorylation and protein synthesis. The inhibition of phosphorylation was measured in kidney homogenate as described by LOOMIS and LIPMANN (20). Protein synthesis was measured in liver slices. The data reproduced are taken from the paper by FRANTZ *et al.* (21). For better comparison the data are given in percentages of normal oxygen uptake, phosphorylation and alanine incorporation.

TABLE 6. FREE ENERGY CHANGE WITH PEPTIDE SYNTHESIS[1] IN WATER, AT 310.5°K

COMPOUND	ΔF_0 CAL.	K (SYN.)
Glycyl-glycine	3070	0.0076
Alanyl-glycine	2590	0.016
Leucyl-glycine	2795	0.012
Glycero-phosphate[2]	2280	0.025

[1] Measurements by Henry Borsook (private communication).

[2] LIPMANN, F. *Advances in Enzymology* 1: 99, 1941.

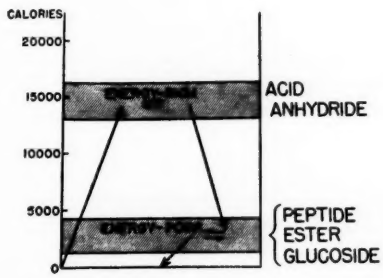


Fig. 5. CHARTING OF RELATIONSHIP between the two energy levels dominant in cellular chemistry and corresponding to energy-rich and energy-poor phosphate bond levels.

reactions on the lower level, as, e. g., ester, peptide and glucoside syntheses. In such rather frequent instances then not more than about a quarter of the available energy is utilized.

This appears, however, not too surprising. The more and more realized applicability in biosynthesis of a standard form of energy for a great variety of purposes has to entail in some instances a more or less pronounced loss of energy, in exchange for a simplification of procedure.

REFERENCES

1. Lipmann, F. *J. Biol. Chem.* 160: 173, 1945.
2. Lipmann, F. *J. Biol. Chem.* 155: 55, 1944.
3. Lipmann, F. *Advances in Enzymology* 1: 99, 1941.
4. Kaplan, N. O. and F. Lipmann. *Federation Proc.* 6: 266, 1947.
5. Lipmann, F. and L. C. Tuttle. *J. Biol. Chem.* 159: 21, 1945.
6. Lipmann, F. and L. C. Tuttle. *J. Biol. Chem.* 161: 415, 1945.
7. Chantrenne, H. *Compt. Rend. Carlsberg Laboratory* 26: 231, 1948.
8. Kaplan, N. O. and M. Soodak. *Federation Proc.* 8: 211, 1949.
9. Speck, J. F. *J. Biol. Chem.* 168: 403, 1947.
10. Elliott, W. H. *Nature* 161: 128, 1948.
11. Elliott, W. H. and E. F. Gale. *Nature* 161: 129, 1948.
12. Cohen, P. P. and R. W. McGilvery. *J. Biol. Chem.* 169: 119, 1947.
13. Lipmann, F. *Advances in Enzymology* 6: 231, 1946.
14. Chou. T. C. and F. Lipmann, Unpublished.
15. Bloch, K. *Abstr. Am. Chem. Soc. Meet.* 46C (Chicago, April 1948).
16. Johnston, R. B. and K. Bloch. *J. Biol. Chem.* 179: 493, 1949.
17. Ivanovics, G. and V. Bruckner. *Ztschr. f. Immunitaets Forsch.* 90: 304, 1937.
18. Frantz, I. D., R. B. Loftfield and W. W. Miller. *Science* 106: 544, 1947.
19. Winnick, T., F. Friedberg, and D. M. Greenberg. *Arch. Biochem.* 15: 160, 1947.
20. Loomis, W. F., and F. Lipmann. *J. Biol. Chem.* 173: 807, 1948.
21. Frantz, I. D., Jr., P. C. Zamecnik, J. W. Reese and M. L. Stephenson. *J. Biol. Chem.* 174: 773, 1948.
22. Axelrod, B. *J. Biol. Chem.* 172: 1, 1948.
23. Borsook, H. in Schmidt, C. L. A. *Chemistry of Amino Acids.* Baltimore: Williams & Wilkins, 1944, p. 865.

MECHANISM OF UREA SYNTHESIS

SARAH RATNER

From the Department of Pharmacology, New York University College of Medicine

NEW YORK CITY

In 1932 Krebs succeeded, for the first time, in demonstrating the *in vitro* formation of urea in mammalian liver. He showed in further detail that the synthesis proceeds by a stepwise mechanism, the ornithine cycle. It is of interest to recent developments in this field that the rapid formation of urea in rat liver slices, as carried out by Krebs and Henseleit (1), required the presence of lactate and oxygen in addition to ornithine, bicarbonate and NH_3.

More recently Cohen and Hayano established the conditions for obtaining arginine synthesis (2) in rat liver homogenates starting with citrulline (Step II of the ornithine cycle), and for obtaining citrulline synthesis (2) from ornithine (Step I). According to their studies of Step II, glutamic acid, rather than NH_3, appeared to be the nitrogen donor in arginine formation from citrulline. Mg ion, catalytic amounts of adenosine triphosphate (ATP) and oxygen were required in addition to the amino acid substrates.

Similar results had been obtained in kidney slices with glutamic and aspartic acid by Borsook and Dubnoff (3) who proposed that the transfer of the $—NH_2$ group occurred by a transamination accompanied by the simultaneous oxidative removal of 2 H atoms (transimination). An α-keto acid, thought to be the second product of the reaction would, of course, escape detection in respiring preparations.

Under the conditions of Borsook and Dubnoff, or of Cohen and Hayano, neither slices nor homogenates can form arginine in the absence of oxygen. Moreover, since the latter group found glutamic acid to be much more effective than aspartic acid in liver homogenates, they considered glutamic acid to be the specific $—NH_2$ donor in the transimination reaction. Aspartic acid was thought to be active only in so far as it could be converted to glutamic acid. We hoped that further insight into the nature of this mechanism might be gained from a study of the isolated system.

By separation and partial purification of the enzymes involved in the citrulline to arginine conversion, it can be shown that aspartic acid is the specific $—NH_2$ donor and that the transfer of nitrogen does not involve an oxidative step. The enzymes concerned are quite soluble and may be readily obtained from extracts of acetone powder mammalian liver. They withstand exposure to alcohol and ammonium sulfate fractionation procedures at low temperature and keep moderately well (4).

Mechanism of Nitrogen Transfer. In the reaction, aspartic acid and citrulline are converted to arginine and malic acid, anaerobically, in the presence of Mg ions. ATP participates directly as a reactant.

As shown in table 1, line 1, 14.4 μM of arginine (estimated as urea) and an equivalent amount of malic acid were formed from aspartic acid and citrulline in 20 minutes by an extract of beef liver acetone powder, subjected to one alcohol fractionation. When either citrulline or aspartic acid was omitted, neither arginine nor malic acid was formed.

Although ATP is specifically required, maximum rates cannot be reached with ATP alone, owing to competition by contaminating ATP-ase and to an inhibition caused by high concentrations of ATP. The difficulty can be overcome by employing 3-phosphoglyceric acid, in excess, as a source of high energy phosphate ($\sim p$H), and ATP in catalytic amounts, to effect the phosphate transfer. The glycolytic enzymes catalyzing the transfer of $\sim p$H through phosphopyruvic acid and ADP are present in the preparation. When necessary their concentration was augmented by addition of a fraction from rabbit muscle extract. The dependence of arginine synthesis upon $\sim p$H is shown in table 1, line 3; when phosphoglyceric acid was omitted, neither arginine nor malic acid was formed.

Glutamic acid could not replace aspartic acid (line 5) nor could any of some 20 other amino acids tried. With glutamic and oxalacetic acids

(line 4) 7 μM of arginine and malate were formed. The activity of this combination is due to the formation of aspartic acid by transamination. Assay of the glutamic-aspartic transaminase content of various preparations shows that the extent to which the combination of glutamic and oxalacetic acids will replace aspartic acid is proportional to the transaminase content of the preparation.

Aspartic acid cannot be replaced by NH_3 or by the combination of oxalacetic acid and NH_3. It is

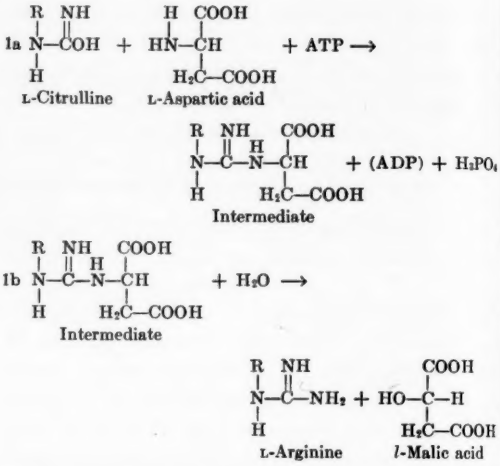
TABLE 1. SYNTHESIS OF ARGININE FROM CITRULLINE IN ALCOHOL FRACTIONATED EXTRACT OF OX LIVER ACETONE POWDER

Each tube contained in addition: 4 μM ATP; 13 μM $MgSO_4$; 0.4 ml. phosphate buffer, *p*H 7.5; enzyme preparation containing 31 mg. protein and 8 mg. muscle extract in a final volume of 4 ml. Time 20 min., 38°.

SUBSTRATE ADDED					FOUND	
L-Aspartate 20 μM	L-Glutamate 20 μM	Oxalacetate 30 μM	L-Citrulline 20 μM	3 Phosphoglycerate 50 μM	Arginine μM	*l*-Malic acid μM
+			+	+	14.4	14.5
+				+	0.0	0.0
+			+		0.9	1.0
	+	+	+	+	7.0	6.8
	+		+	+	0.0	0.0
	+	+		+	0.0	0.1
		+	+	+	0.0	0.2

particularly significant that in the absence of citrulline, no combination including oxalacetic acid gave rise to malic acid. For example, with oxalacetic and glutamic acids (line 6), malic acid would certainly have been formed by a glutamic dehydrogenase–malic dehydrogenase catalyzed dismutation (5) had diphosphopyridine nucleotide (DPN) been present. Under the given experimental conditions, malic acid cannot be formed independently of arginine.

The simultaneous formation of arginine and malic acid, by interaction of the two substrates, strongly suggests that the mechanism of nitrogen transfer involves a preliminary condensation of the amino group of aspartic acid with the ureido group of citrulline to form a C—NH—C linkage, followed by hydrolytic cleavage on the second side of the nitrogen, as shown in reactions 1a and 1b.



Regarding optical specificity of the overall reaction, only L-citrulline and L-aspartic acid react, while *l*-malic acid and L-arginine are formed. Identification of the products of the reaction has been established by isolation from a large-scale enzymatic run. *l*-Malic acid was isolated as the cinchonine salt and estimated quantitatively with the highly purified 'malic enzyme' described by Ochoa, Kornberg and Mehler (6). Since arginase was always present in excess, urea was estimated by the colorimetric method of Archibald (7) and L-ornithine was isolated as the dibenzoylderivative.

Intermediary Condensation Product. The intermediary condensation product shown in reaction 1a is quite different from that postulated in keto-amino acid transimination. In the latter case, the reaction, however formulated (8–10), supposedly occurs through transient Schiff base formation, and the whole reaction is catalyzed by a single enzyme. Here the condensation product is depicted as an N,N′ disubstituted guanidine and at least two enzymatically distinct steps are involved in the overall synthesis of arginine.

The two enzymes have been separated by ammonium sulfate fractionation of extracts of ox liver acetone powder, thus permitting a study of each step separately. In the first step (reaction 1a), ATP and Mg ion are specifically required. Urea formation from CO_2 and NH_3 is known to be endergonic. It may therefore be reasonably assumed that both citrulline and arginine synthesis are each endergonic, and that as far as arginine synthesis is concerned, it is precisely in the condensation reaction, that energy, supplied by ATP, is actually utilized.

Presumably, condensation occurs via a pre-

liminary phosphorylation. Although much information regarding the phosphorylation step is still lacking, phosphorylation of the isourea form of citrulline rather than of aspartic acid would appear more likely, for it would provide a means of shifting the citrulline equilibrium toward the more reactive isourea form.

Table 2 shows some of the experimental evidence for the stepwise reaction. Since phosphoglyceric acid was used as the source of ~*p*H in the presence of condensing enzyme A, the amount of pyruvic acid formed, as well as the amount of inorganic phosphate, is a measure of the extent of ~*p*H transfer. Citrulline disappear-

TABLE 2. STEPWISE ENZYMATIC FORMATION OF ARGININE

The conditions are as in table 1, line 1, except that muscle extract was omitted and glycylglycine buffer was substituted for phosphate. In the first step a solution of enzyme A containing 25 mg. of protein was added to each tube. 38°, 20 min. After heat inactivation, enzyme B (18 mg. protein per tube) was added. 38°, 20 min. The values are corrected for the small amounts of pyruvate and inorganic phosphate formed in the absence of citrulline.

	ENZYME A μM	ENZYME B μM
Citrulline removed	11.0	
Pyruvate formed	15.5	
Inorganic P formed	17.4	
Arginine formed	0.2	6.1
Malate formed	0.3	6.3

ance was found to be roughly equivalent to the amount of pyruvate and inorganic phosphate formed. The excess of these two over the amount of citrulline removed is undoubtedly due to competition with some contaminating ATP-ase. Neither arginine nor malic acid was formed, but the condensation product appeared to accumulate, for upon addition of the hydrolyzing enzyme B to the deproteinized reaction mixture, arginine and malic acid were formed in equivalent amounts.

About 50 mg. of the intermediate have been accumulated in a large scale enzymatic run with enzyme A. By fractionation of the barium salt with alcohol, the intermediate has been freed of organic and inorganic phosphorous compounds originally present in the medium, and of citrulline; though traces of aspartic and pyruvic acids remain. At this stage of purification, enzymatic cleavage to arginine and malic acid requires no further additions beyond enzyme B and the intermediate, thus confirming the formulation of reaction 1b as being purely hydrolytic.

Comparison of Arginine Synthesis in Slices, Homogenates, and Extracts. A comparison of the specific activity, expressed as Q_{urea} (μl. arginine or urea formed per mg. protein per hour), of various liver preparations is given in table 3. The activity of crude extracts of ox liver acetone powder varies with the age of the powder. A preparation made from a powder after 4 weeks' storage had a Q_{urea} of 7 which was increased to 11 by the addition of an extract of rabbit muscle, thus accelerating the rate of arginine synthesis to the point where only arginine synthesizing enzymes were limiting. On alcohol fractionation, a three-fold purification was reached in fraction 4 (Q_{urea} 33 with added muscle extract) corresponding to about 30 per cent alcohol.

TABLE 3. SPECIFIC ACTIVITY OF VARIOUS LIVER PREPARATIONS

SPECIES	PREPARATION	CONDITIONS	Q_{UREA}
Rat	Slice	NH_3, Lactate	18
	Homogenate	Glutamate[2]	14
	Homogenate	Aspartate, pyruvate[3]	16
	Acetone powder extract	Aspartate, ~ *p*H	18.8
	Acetone powder extract	Aspartate, ~ *p*H + muscle extr.	20.2
Ox	Acetone powder extract	Aspartate, ~ *p*H	7.4
	Acetone powder extract	Aspartate, ~ *p*H + muscle extr.	11.2
	EthOH fraction 3	Aspartate, ~ *p*H	7.8
	EthOH fraction 3	Aspartate, ~ *p*H + muscle extr.	20.0
	EthOH fraction 4	Aspartate, ~ *p*H	17.9
	EthOH fraction 4	Aspartate, ~ *p*H + muscle extr.	33.6

[1] Q_{urea} recalculated from the data of Krebs and Henseleit, assuming 85 % of the dry weight to be protein. [2] Calculated from the rate of arginine synthesis per mg. N given by Cohen and Hayano. [3] Calculated from the data in table 4, assuming 85% of the dry weight to be protein.

Though much less stable, extracts of rat liver acetone powder have a higher specific activity. Under optimal conditions a Q_{urea} of 20 was found as compared to a Q_{urea} of 18 obtained by Krebs for rat liver slices, and a Q_{urea} of 14 obtained by Cohen and Hayano for rat liver homogenates in the presence of glutamic acid. In the presence of aspartic acid, supplemented with pyruvic acid,

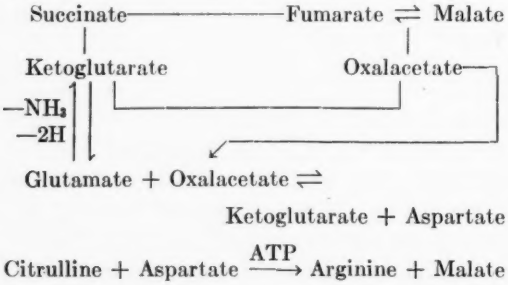
rat liver homogenates gave a somewhat higher Q_{Urea} value of 16.

On calculating the total activity of an extract of rat liver acetone powder, it appears that about 50 per cent of the original arginine synthesizing activity of the whole tissue can immediately be accounted for in the extract, without correction for destruction by acetone treatment or incompleteness of extraction, suggesting that the same enzyme system is involved regardless of the substrates offered or of other variations in experimental conditions.

RELATIONSHIP OF THE TRICARBOXYLIC CYCLE AND OF TRANSAMINASE TO ARGININE SYNTHESIS

If, as studies on the anaerobic isolated system indicate, aspartic acid is the specific $—NH_2$ donor in arginine synthesis, then a number of questions are raised in relation to published observations concerning the following main points. *1*) Glutamic acid is four times as effective as aspartic acid in homogenates, aerobically (2, 11). *2*) Both glutamic acid and aspartic acid are poor precursors in liver slices (1, 2). *3*) The general mechanism of nitrogen transfer from amino acids to form aspartic acid, prior to arginine synthesis, is not immediately apparent.

With regard to glutamic acid, an outline of the proposed enzymatic pathway is shown below where the rectangle is meant to represent the Krebs tricarboxylic cycle in highly schematized form, including only those intermediates of the cycle pertinent to the present discussion.



$$\text{Citrulline} + \text{Aspartate} \xrightarrow{\text{ATP}} \text{Arginine} + \text{Malate}$$

In homogenates, aerobically, when glutamate is the $—NH_2$ donor, some of the glutamic acid is oxidized through glutamic dehydrogenase and the tricarboxylic cycle, to form oxalacetate. The latter is then converted to aspartate by transamination with a further amount of glutamate. ATP, generated by phosphorylations coupled with oxidation, is formed during these steps. From a quantitative point of view, it has been shown by Ochoa (12) that the ratio of $\sim p_H$ generated per atom of oxygen is 3:1; thus more than an ample supply would be provided for arginine synthesis. The oxygen requirement in liver homogenates, when glutamate is the NH_2 donor, must then be ascribed to dependence upon *a*) ATP generation and *b*) oxalacetate formation.

When aspartate is the NH_2 donor, in homogenates, arginine synthesis is poor because aspartic acid is oxidized very slowly and little, if any, oxalacetate is released to enter the tricarboxylic cycle. Sufficient ATP for arginine synthesis is therefore not made available, it being supplied directly only in catalytic amounts. If aspartic acid is supplemented by pyruvate, α-ketoglutarate, or any other member of the tricarboxylic cycle, arginine synthesis is increased to a high value. The values for oxygen uptake and arginine synthesis from aspartic acid, with and without the addition of various respiratory substrates, are given in table 4.

If when glutamate is offered aspartate must be formed by transamination, prior to arginine

TABLE 4. SYNTHESIS OF ARGININE FROM CITRULLINE IN RAT LIVER HOMOGENATES UNDER AEROBIC CONDITIONS IN THE ABSENCE AND PRESENCE OF MALONATE

In addition each vessel contained: 20 μM L-citrulline; 5 μM ATP; 10 μM $MgSO_4$; 0.3 ml. of 0.25M potassium phosphate, p_H 7.5; 0.5 ml. of 25 % homogenate. Final volume 3.0 ml., 38°, time 40 minutes. Calculated for 31 mg. dry weight of tissue. The values are given in μM.

SUBSTRATE ADDED 20 μM	WITH L-ASPARTATE				WITH L-GLUTAMATE			
	Arginine		O_2 uptake		Arginine		O_2 uptake	
	av.	a.d.[1]	av.	a.d.	av.	a.d.	av.	a.d.
None	3.5	±0.8	14.9	±0.2	9.6	±1.1	30.5	±2.7
Pyruvate[2]	12.8	±0.7	29.4	±1.2	6.0	±1.0	30.5	±1.8
3-phosphoglycerate	12.9	±0.9	28.9	±1.2	6.2	±1.1	22.1	±5.5
Oxalacetate	14.4	±0.4	30.1	±1.9	10.9	±1.2	28.8	±5.0
Fumarate	13.4	±1.0	27.4	±1.6	12.5	±0.8	30.8	±2.4
α-ketoglutarate	11.1	±0.4	23.7	±3.9	4.4	±0.7	23.1	±3.1
With 60 μM of malonate per vessel								
None	3.3	±1.0	10.8	±2.0	2.6	±0.0	18.9	±0.6
Pyruvate	11.4	±0.2	19.9	±1.3	2.6	±0.4	19.1	±1.4
2-phosphoglycerate	11.8	±1.3	21.5	±2.3	2.3	±0.3	17.7	±2.0
Oxalacetate	13.3	±0.4	24.1	±1.9	13.3	±0.3	27.4	±2.6
Fumarate	13.3	±0.5	25.4	±1.6	12.8	±1.0	28.1	±3.7
α-ketoglutarate	11.8	±0.1	19.8	±2.7	1.5	±0.1	13.8	±1.9

[1] Average deviation. [2] 2.5 μM fumaric acid was added as a primer. Good respiration was often obtained without it, but the addition insured uniformly high values. Fumarate was not added to pyruvate when glutamate was employed, nor was it added in any malonate experiment.

synthesis, then it may be anticipated that whatever affects the concentration of aspartic acid, either directly through the transaminase equilibrium or secondarily, will affect the rate of arginine synthesis.

Certain inhibitory effects reported from the laboratories of Borsook, Cohen, Krebs and Leuthardt may be explained on this basis. For example, the addition of α-ketoglutarate to glutamate (2, 11, 13) inhibits the rate of arginine synthesis about 40 to 60 per cent by pushing the transaminase equilibrium to the left, thus lowering the aspartate concentration (table 4).

Pyruvate exerts a similar inhibition (2, 3, 11, 13); by transamination with glutamic acid, some glutamate is removed and α-ketoglutarate formed. Both of these effects tend to diminish the aspartic acid concentration (table 4).

When aspartate is offered directly as the $—NH_2$ donor, the aspartate concentration is in excess, as far as enzyme saturation is concerned. Even if lowered from 20 to 15 μM by the addition of 20 μM of α-ketoglutarate, arginine synthesis will still be quite rapid (table 4).

Malonate also inhibits arginine synthesis from glutamate (2, 11, 14). This can readily be explained by the well-known block of α-ketoglutarate oxidation at the succinic dehydrogenase step, thus preventing the formation of oxalacetate. This explains why the addition of the latter or of a closely related precursor, such as fumaric or malic acids (14), specifically removes the inhibition (table 4). As is to be expected, the addition of malonate has practically no effect on arginine synthesis from aspartate under optimal conditions (*i.e.* in the presence of a respiratory substrate), for the rate of ATP generation, while excessive in the absence of malonate (see values for O_2 uptake in table 4) is sufficiently rapid even in the presence of malonate, to maintain a high rate of arginine synthesis.

Anaerobic Synthesis of Arginine in Homogenates. Once the requirements of the system are known, it is possible to obtain arginine synthesis in homogenates under anaerobic conditions by utilizing glycolytic reactions to generate $\sim p_H$. Table 5 shows the amount of arginine formed in the presence of citrulline, aspartic acid and phosphoglyceric acid or hexose diphosphate. None was formed in the absence of a supply of $\sim p_H$. The addition of phosphoglyceric acid, or of hexose diphosphate, caused no arginine formation when glutamate was substituted for aspartate, unless oxalacetate was also present.

Owing to the decreased efficiency of anaerobic, as compared to aerobic phosphorylation, competition with ATP-ase is less successful anaerobically. The rates of arginine synthesis were therefore much lower, anaerobically, than those obtained with an equal amount of homogenate in oxygen. A considerable portion of the ATP-ase present is associated with the suspended particles of the homogenate, and can be removed by high speed centrifugation. The last column of table 5 gives the values obtained in an equivalent amount of supernatant, after removing the particles. Arginine when formed was found to be higher than in the whole homogenate in each case.

TABLE 5. SYNTHESIS OF ARGININE FROM CITRULLINE IN RAT LIVER HOMOGENATES UNDER ANAEROBIC CONDITIONS

Further additions and other conditions as in table 4 except that N_2 replaced O_2 in the gas space and 4 mg. DPN was added to each vessel containing hexosediphosphate. The dry weight of 0.5 ml. of homogenate and 0.4 ml. of supernatant respectively were 34.7 mg. and 17.1 mg. The values are given in μM.

SUBSTRATE ADDED					ARGININE FOUND	
L-Aspartate 20 μM	L-Glutamate 20 μM	Oxalacetate 30 μM	3 Phosphoglycerate 40 μM	Hexose diphosphate 25 μM	Whole homogenate	Supernatant
+			+		3.9	7.7
+				+	5.7	8.0
+					0.6	1.2
	+		+		0.1	0.0
	+			+	0.5	0.4
	+	+	+		4.6	6.8
	+	+		+	4.2	4.9

These experiments parallel the behavior of extracts of acetone powder. From the data in table 5, it may be seen that both the arginine synthesizing enzymes and transaminase are present in the supernate. This further supports the view that the particles were required only for the generation of ATP and of oxalacetate, when glutamate was the $—NH_2$ donor in the aerobic experiments.

Transfer of Amino Nigrogen to Oxalacetate. As to the general problem of $—NH_2$ transfer to form aspartic acid, tissue slice, isolated enzyme and isotope evidence (15–17) indicate that a good part of amino acid nitrogen probably goes through NH_3. However, there appears to be no known en-

zyme which oxidizes aspartic acid rapidly, comparable for example to glutamic dehydrogenase in activity and reversibility. Nevertheless rapid nitrogen transfer is possible by means of a combination of glutamic dehydrogenase and glutamic-aspartic transaminase, particularly under conditions where the newly formed aspartate can be continuously removed.

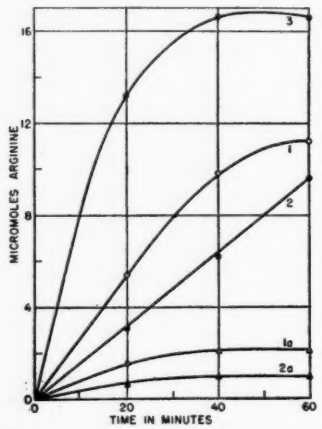


FIG. 1. TRANSFER of NH_3 to citrulline through aspartic acid by anaerobic dismutation and transamination in ox liver acetone powder extracts. Curve 1: malate, α-ketoglutarate dismutation. Curve 1a: same as curve 1 in the absence of either DPN, NH_3 or malate. Curve 2: triosephosphate, α-ketoglutarate dismutation. Curve 2a: same as curve 2 in the absence of either DPN, NH_3 or oxalacetate. Curve 3: arginine formation with aspartate in the same amount of extract. All tubes contained 4 μM ATP; 20 μM L-citrulline; 13 μM $MgSO_4$; 0.4 ml. of 0.25 M potassium phosphate *p*H 7.5 and 1.0 ml. acetone powder extract in a final volume of 4 ml.; temperature 38°. Further additions were: curve 1: 20 μM each NH_4Cl and *l*-malate, 10 μM α-ketoglutarate, 50 μM 3-phosphoglyceric acid and 2 mg. DPN. Curve 2: 20 μM NH_4Cl, 10 μM α-ketoglutarate, 30 μM oxalacetate, 25 μM hexosediphosphate and 2 mg. DPN. Curve 3: 20 μM L-aspartate, 50 μM 3-phosphoglycerate.

The transfer of NH_3 to form aspartic acid has been carried out in extracts of ox liver acetone powder which contain these enzymes by means of dismutation reactions coupled with transamination.

1. Malic acid + DPN_{ox} $\rightleftharpoons$ oxalacetic acid + DPN_{red}

1a Triosephosphate + H_3PO_4 + DPN_{ox} $\rightleftharpoons$ diphosphoglyceric acid + DPN_{red}

2. NH_3 + α-ketoglutaric acid + DPN_{red} $\rightleftharpoons$ glutamic + DPN_{ox}

3. Glutamic acid + oxalacetic acid $\rightleftharpoons$ α-ketoglutaric acid + aspartic acid

4. Malic acid + NH_3 $\rightleftharpoons$ aspartic acid

4a Triose phosphate + H_3PO_4 + NH_3 + oxalacetic acid $\rightleftharpoons$ diphospho glyceric acid + aspartic acid

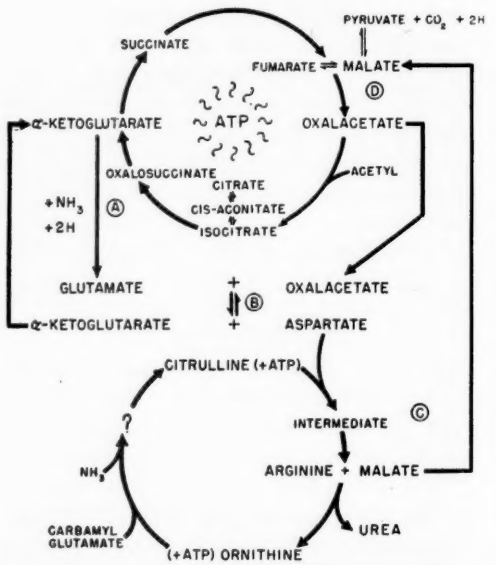


FIG. 2. PATHWAY OF AMINO NITROGEN TRANSFER to citrulline in urea synthesis and the relationship of the ornithine cycle to the tricarboxylic cycle.

In the first dismutation, malic acid undergoes a DPN linked dismutation with NH_3 and α-ketoglutarate, catalyzed by malic and glutamic dehydrogenases (reactions 1 and 2) resulting in the formation of oxalacetate and glutamate, which then transaminate (reaction 3) resulting in aspartate formation (reaction 4). In the presence of phosphoglycerate and citrulline, the amount of arginine formed becomes a measure of the rate of aspartate synthesis (fig. 1, curve 1).

In the second dismutation, triosephosphate dehydrogenase (reaction 1a) replaced malic dehydrogenase. Starting with NH_3, α-ketoglutarate, oxalacetate and hexosediphosphate (as the source of triosephosphate), aspartate will be formed as shown by reaction 4a. With the resulting diphosphoglycerate as the source of $\sim p$H, arginine was formed as shown in curve 2. The rate of arginine

formation in an equal amount of extract with aspartate and phosphoglycerate is shown in curve 3. Curves 1a and 2a represent blank values for the dismutations in the absence of NH_3 or DPN.

PHYSIOLOGICAL PATHWAY OF AMINO NITROGEN TRANSFER IN UREA FORMATION

The individual steps in the aerobic transfer of nitrogen to form urea and their relationship to transaminase and to the tricarboxylic cycle of Krebs are summarized in figure 2.

Citrulline formation from ornithine (Step E), which appears to require ATP generated by the tricarboxylic cycle, proceeds with carbamyl glutamate as the source of the ureido carbon, according to the most recently published work of Cohen and Grisolia (18). Citrulline is then converted to arginine (Step C) by a two-step reaction involving ATP (generated oxidatively) and aspartate, in the formation of an intermediate, which is then split to form arginine and malic acid.

In homogenates with glutamate as the $—NH_2$ source, the transfer of nitrogen will start by transamination (Step B) with oxalacetate supplied by respiration.

In slices, NH_3 transfer will occur at Step A (glutamic dehydrogenase) and Step E. It has become apparent from recent studies on the permeability of liver slices that the poor activity of aspartic and glutamic acids is due to their slow rate of permeability. In the intact animal, NH_3 arising from amino acid deamination will enter the cycle as with slices.

The scheme represents an expansion of the ornithine cycle of Krebs, connected with the Krebs tricarboxylic cycle by transaminase and by two simpler cycles; one which permits the catalytic turnover of α-ketoglutaric acid as a carrier of NH_3 by means of glutamic dehydrogenase; and the other which permits the malic acid (formed as the second product in arginine synthesis) to function, catalytically, in aspartic acid formation by means of malic dehydrogenase.

From studies on the isolated system and on homogenates, the scheme shown here appears to represent a major pathway of urea formation and accounts for most of the observations on slices and homogenates reported from other laboratories.

REFERENCES

1. Krebs, H. A., and K. Henseleit. *Ztschr. f. physiol. Chem.* 210: 33, 1932.
2. Cohen, P. P., and M. Hayano. *J. Biol. Chem.* 166: 239, 251, 1946; 172: 405, 1948.
3. Borsook, H. and J. W. Dubnoff. *J. Biol. Chem.* 141: 717, 1941.
4. Ratner, S., and A. Pappas. *J. Biol. Chem.* 179: 1183, 1199, 1949.
5. Dewan, J. G. *Biochem. J.* 33: 549, 1939.
6. Ochoa, S., A. H. Mehler, and A. Kornberg. *J. Biol. Chem.* 174: 979, 1948.
7. Archibald, R. M. *J. Biol. Chem.* 157: 507, 1945.
8. Braunstein, A. E., and M. G. Kritzman. *Enzymologia* 2: 129, 1937.
9. Schlenk, F., and A. Fischer. *Arch. Biochem.* 12: 69, 1947.
10. Umbreit, W. W., D. J. O'Kane, and I. C. Gunsalus. *J. Biol. Chem.* 176: 629, 1948.
11. Krebs, H. A., and L. V. Eggleston. *Acta biochim. et biophys.* 2: 319, 1948.
12. Ochoa, S. *J. Biol. Chem.* 138: 751, 1941; 151: 493, 1943.
13. von Fahrlander, H., H. Nielson, and F. Leuthardt. *Helv. Chim. Acta* 31: 957, 1948.
14. von Fahrlander, H., P. Favarger, and F. Leuthardt. *Helv. Chim. Acta* 31: 942, 1948.
15. Krebs, H. A. *Biochem. J.* 29: 1620, 1935.
16. Blanchard, M., D. E. Green, V. Nocito, and S. Ratner. *J. Biol. Chem.* 155: 421, 1944.
17. Schoenheimer, R. *The Dynamic State of Body Constituents.* Harvard University Press: Cambridge, 1942.
18. Cohen, P. P., and S. Grisolia. *J. Biol. Chem.* 174: 389, 1948.

American Institute of Nutrition

Symposium on Nutrition in Preventive Medicine

Chairman: C. G. KING

NUTRITION, RENAL LESIONS AND HYPERTENSION[1]

CHARLES H. BEST AND W. STANLEY HARTROFT

From the University of Toronto

TORONTO, CANADA

PREVENTION of the accumulation of excess fat in the liver by dietary choline was demonstrated in our laboratories in 1932 using depancreatized dogs and normal rats (1). In 1939, Griffith and Wade (2) reported that in addition to fatty livers, weanling rats exhibited a fulminating, hemorrhagic lesion of the kidneys (fig. 6). The occurrence of this syndrome was subsequently confirmed by György and Goldblatt (3), by Engel and Salmon (4) and by Handler (5), and it has also been extensively studied in our laboratory. In 1947, Sobin and Landis (6) demonstrated that the blood pressure remained normal in young rats maintained on diets deficient in choline for as long as 7 months. We have confirmed these results by direct measurements in weanling rats exhibiting the syndrome of acute choline deficiency, and also in older animals which were given similar diets for longer periods. As an extension of these investigations, we have also studied the blood pressures in a third group of rats. These were fed the diet low in choline for 6 days at ages of 3 to 4 weeks, and this was then replaced by a fully normal food mixture for the remainder of the experimental period of 4 to 7 months. Of these animals, more than one-third developed a moderate or severe degree of hypertension during the period of observation.

RENAL LESION PRODUCED IN WEANLING RATS BY DIETARY CHOLINE DEFICIENCY

A brief outline of the changes which occur in the kidneys of weanling rats during the acute stage of choline deficiency will be presented, for these lesions are presumably responsible for the elevation in the level of blood pressure which later develops in many of those animals which survive this initial period. Christensen (7) working with Griffith and Wade reported the pathology of the hemorrhagic renal syndrome. On gross examination, the kidneys are swollen and red and may attain weights twice the normal (fig. 6). Christensen's histological investigations demonstrated the presence of a widespread tubular lesion throughout the renal cortices, associated with hemorrhage into and beneath the capsule (fig. 7). Tubules in the outer portion of the cortex were necrotic and those lying more deeply were degenerated (fig. 8). Hyaline casts filled the tubules at the cortico-medullary junction (fig. 9). Stainable fat droplets were present in some of the cortical tubules. These observations were confirmed by György and Goldblatt (3) who also emphasized the primary nature of the tubular lesions, for glomerular degeneration always followed the tubular necrosis as a secondary event.

The pathogenesis of these lesions has been the subject of detailed investigations in our laboratories (8). It was shown that the initial lesion demonstrable in the kidneys of weanling rats fed diets low in choline consists of the formation of small droplets of stainable fat within the epithelial cells of the proximal convoluted tubules of the cortex (figs. 10 and 11). These droplets appear within 2 to 5 days of dietary choline deficiency. This is associated with generalized swelling of the affected nephrons. Evidence has been presented (8) which indicates that tubular swelling produces obstruction of the intervening cortical capillary plexus with resultant tubular ischemia and necrosis (figs.

[1] Presented as part of the Symposium on Nutrition in Preventive Medicine, Detroit, Mich., April 21, 1949.

1 and 2). This mechanism has been termed tubular obstruction of capillaries (TOC). Proximal to the sites of capillary obstruction, the cortical vessels become engorged and rupture into and beneath the renal capsule (fig. 7). This combination of parenchymal swelling and necrosis with associated capsular hemorrhage is responsible for the gross appearance of the kidneys. The development of this lesion is necessarily accompanied by a reduction of the volume and extent of the renal vascular plexus in the cortex. As glomeruli are destroyed in the later stages, their total numbers are accordingly reduced.

PRODUCTION OF HYPERTENSION IN SURVIVORS OF THE HEMORRHAGIC RENAL SYNDROME

Methods. Weanling albino rats of the Wistar strain (35–42 gm.) were fed a diet very low in choline and its precursors.[2] The object at this stage of the experiment was to produce the greatest degree of renal damage compatible with life. A series of preliminary trials indicated that the optimum duration of dietary choline deficiency for this purpose was only 5 or 6 days. Pair-fed control animals received the same basal diet supplemented with 0.35 per cent choline chloride. At the end of this short period of choline deficiency, the experimental group and also the controls were given a good stock diet which allowed excellent growth and development throughout the remaining 4 to 7 months of the experiment. During this period, indirect determinations of the blood pressures of all animals were made at intervals by the method of Sobin (9). But the final assessment of arterial tension was based on direct measurement of blood pressure as indicated by a fine-bore mercury manometer connected with a 24-gauge needle inserted into the animal's femoral artery. Following exsanguination and death, the heart was weighed to the nearest 1/100 gram in each case. For comparative purposes, heart weight was expressed as a percentage of the total body weight. Paraffin sections of kidneys, heart and other viscera were stained by a variety of methods. On an histological basis, the animals were tabulated in three groups according to the amount of residual renal damage observed in microsections: *slight*, *moderate* or *severe*. The series included 62 animals which had survived varying degrees of the renal hemorrhagic syndrome and 36 controls of similar age and weight. The controls had received the same diet as the test animals throughout the entire experiment, but were given a choline supplement during the initial 5- to 6-day period when the experimental groups were deprived of lipotropic factors.

Results. These are presented in graphic form (fig. 5). Ten of the 62 experimental animals had suffered *severe* residual renal damage as demonstrated by microscopic examination. The average blood pressure of this group was 195 mm. Hg (systolic-diastolic mean) and their heart weights were nearly double the normal. Thirteen rats had *moderate* degrees of renal damage, and of these, 11 had blood pressures of 150 mm. Hg or over and were thus considered hypertensive; the average for the group was 165 mm. Hg. The average weight of the hearts of the animals which comprised this second group was approximately one-third greater than that of the controls (expressed as percentage of body weight). The third group, which consisted of the remaining 39 experimental rats, had suffered only *slight* degrees of residual renal damage, and the average of the levels of blood pressure in these animals was only a little higher than for the controls. The blood pressures of all these control animals, with one exception, were lower than 140 mm. Hg. The average figure was 118 mm. Hg.

The microscopic appearance of kidneys with

---

[2] Arachin, 12%; Gelatin, 6%; Casein, 3%; Fibrin, 1%; Salts,* 5%; Celluflour, 2%; Sucrose, 57.5%; Vitamin powder,** 1%; Beef fat, 10%; Corn oil, 2%; Cod liver oil concentrate,*** 0.015%; Cystine, 0.50%

* Salts: composition/100 gm.
Calcium lactate, 35.00 gm.
Calcium carbonate, gm. 5.06
$Ca(H_2PO_4)_2 \cdot H_2O$ 14.60 gm.
K_2HPO_4, 6.46 gm.
$NaH_2PO_4H_2O$, 18.76 gm.
NaCl, 9.34 gm..
$MgSo_4$ (anhydrous), 7.18 gm.
Fe citrate, 3.20 gm.
and trace salts:
$MnSO_4 \cdot 4H_2O$, 0.33 gm.
$ZnSO_4 \cdot 7H_2O$, 0.035 gm.
$CuSO_4 \cdot 3H_2O$, 0.039 gm.
KI, 0.00039 gm.

**Vitamin powder/100 gm. mixture
Thiamin, 500 mg.
Riboflavin, 250 mg.
Pyridoxine, 200 mg.
Calcium pantothenate, 1 gm.
Nicotinic acid, 1 gm.
Added to powdered sugar, 997.05 gm.

***Cod liver oil concentrate used, contains 50,000 I.U. of Vit. D. and 200,000 I.U. of Vit. A/gram. Supplied by Messrs. Ayerst, McKenna and Harrison, Montreal, Canada.

severe degrees of damage indicated extensive loss of both tubules and glomeruli (fig. 12). Those which had escaped destruction were often greatly enlarged. Casts filled most of the distended tubules (fig. 13). Hyalinization in varying degrees was encountered in many glomeruli (figs. 16 and 17). Preparations injected with india ink demonstrated that the glomerular capillary bed was most severely reduced in those animals which had developed the greatest elevation in blood pressure (figs. 3 and 4). Arterial lesions were present which were very similar in nature to those which are associated with both benign and malignant phases of human hypertension (figs. 18 to 21). In two of 10 rats with arteriolar necrosis, the level of nonprotein nitrogen in the blood was determined and found in both instances to exceed 350 mg. per cent. No evidence of inflammatory disease could be demonstrated in sections of any of the hypertrophied hearts of the hypertensive animals.

Discussion. The renal lesions of the hypertensive animals were the result not only of the acute period of choline deficiency in early life, but probably also of the hypertension sustained during the last few weeks before death. Such features as hyalinization of glomerular loops and thickening of Bowman's capsule are, in our opinion, most likely the result of high levels of intravascular pressure. On the other hand, parenchymal loss is probably attributable to the preliminary period of acute dietary deficiency. Experiments are now in progress, the results of which should help us to obtain a better understanding of the pathogenesis of these renal lesions.

It has been suggested by Dr. Handler (10) that fibrotic organization in the areas of capsular and subcapsular hemorrhages, which occur in the acute initial period of deficiency, might be a factor in the production of hypertension later in these animals. Such a mechanism would be analagous to that which operates in rats which develop high levels of blood pressure after the kidneys have been wrapped with cellophane or silk (11) (fig. 14). In a few of our animals, very slight proliferation of fibrous tissue had occurred in capsular areas (fig. 12), but in most cases there was no evidence of abnormal amounts (fig. 15). In the acute period of renal damage, however, the site of greatest necrosis is just beneath the capsule. It is possible that this necrotic tissue may act like a thickened capsule, but until the entire problem of the pathogenesis of these kidney changes has been more intensively studied, further discussion along these lines will not be attempted.

The reduction of the renal cortical vasculature which has been demonstrated is probably a factor of prime importance in the development of hypertension in these experiments. This would be comparable to that type of hypertension which develops in rats which have been subtotally nephrectomized (12). We have no evidence regarding the hormonal mechanisms involved, but presumably they would be similar to those operating in animals rendered hypertensive by the Goldblatt technique (13).[3]

Initial attempts to produce hypertension in rats by allowing them to survive the hemorrhagic renal syndrome of choline deficiency met with little success. Large numbers of weanling animals were fed the deficient diet for periods as long as 10 days. Only a few which were very resistant to this type of renal lesion survived. Irreversible damage to the kidneys of a degree sufficient to influence the level of blood pressure in later life had apparently not been produced in these few hardy survivors, for hypertension did not develop and histological examination of the kidneys revealed few abnormalities. In later experiments a greater measure of success was obtained by restricting the length of time the young animals were fed the low-choline diet to periods of only 5 or 6 days. The greater number of animals which then survived included many with extensive renal damage. A significant proportion of these survivors later developed hypertension when fed the normal food mixture.

In the experiments conducted by Sobin and Landis (6) in which animals were deprived of dietary choline for long periods, the experimental conditions would also automatically tend to eliminate those rats which were most susceptible to this type of kidney damage. Furthermore, the growth of survivors of the acute period of deficiency when left on low-choline diets, is less than if they are transferred to normal rations such as we have used. Slower growth rats of survivors would be associated with lesser demands on the cardiovascular-renal systems in these animals, and this taken together with only slight degrees of initial kidney damage would explain the apparent

[3] We have seen the hemorrhagic renal syndrome in an adult rat which after a period of undernutrition was placed on a choline-deficient diet. We have not yet learned how to produce these lesions consistently in adult rats.

discrepancies between our results and those of Sobin and Landis. Dr. Handler who has confirmed our findings (10) has suggested a further explanation. He feels that to produce hypertension, it is necessary to feed the survivors a diet which contains a greater percentage of protein than is contained in most choline-deficient food mixtures. Our stock diets employed in the second phase of the experiments contained a normal amount of protein, which was of course a higher level than that in the basal choline-deficient ration.

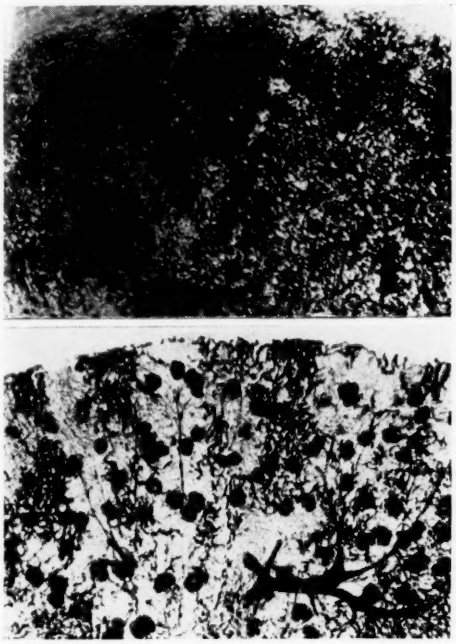


FIG. 1 (*upper*). APPEARANCE OF CORTICAL CAPILLARY PLEXUS in kidney of a normal weanling rat used as control. The vascular system of the rat was injected with a filtered dilution of india ink introduced under physiological pressure into heparinized, anesthetized animal via left ventricle. Slices of formalin-fixed kidney, 100μ in thickness, were dehydrated in ascending strengths of alcohol and cleared in benzyl benzoate. The dense plexus is so well injected that glomeruli are obscured.

FIG. 2 (*lower*). APPEARANCE OF CORTICAL CAPILLARY PLEXUS in kidney of a weanling rat fed basal diet deficient in choline for a period of 5 days. The technique of demonstration was identical with that described above for fig. 1. Note failure of injection mass to penetrate terminal portions of plexus which supply the periphery of each renal lobule. In stained sections, the tubules in the ischemic regions were necrotic. The mechanism by which this type of regional capillary ischemia is produced has been designated TOC—tubular obstruction of the capillaries. The evidence, which has been presented in detail elsewhere (8), indicates that initial lesion in development of hemorrhagic renal syndrome consists of appearance of stainable fat in epithelium of the tubules which, at the same time, become swollen. Pressure is thus exerted on capillaries which lie between affected tubules and results in vascular occlusion—TOC.

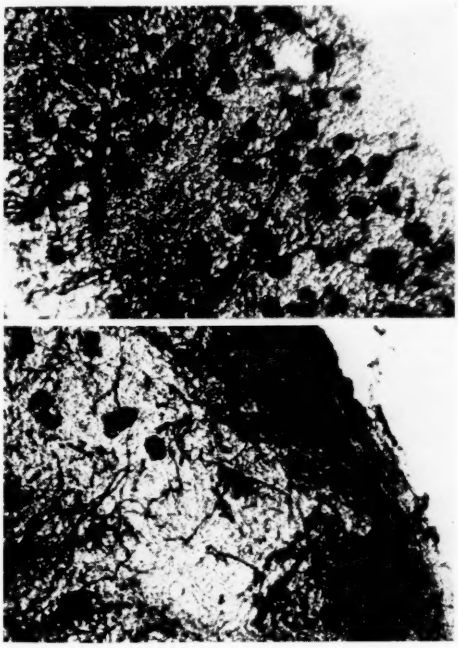


FIG. 3 (*upper*). APPEARANCE OF CORTICAL CAPILLARY PLEXUS in kidney of an adult rat which belonged to the control group. With age, tubules have increased in size so that capillary plexus is separated and not sufficiently dense to obscure glomeruli which also are larger than those in weanling rat illustrated in fig. 1. Techniques employed for preparing and photographing the specimen are the same as those outlined for fig. 1.

FIG. 4 (*lower*). APPEARANCE OF CORTICAL CAPILLARY PLEXUS in kidney of an adult rat which developed a malignant type of hypertension. The animal belonged to the experimental group which was fed choline-deficient diet for 6 days followed by a full normal diet until time of death several months later. Note obvious reduction in number of glomeruli. Technical details are the same as described for fig. 1.

Selye (14) Krakower and Heino (15) and Lenel *et al.* (16) have demonstrated the interrelated roles of the adrenal cortex, the kidney and of dietary salt in the production of hypertension in chickens. Deane and Oleson (17) have shown that there is hypertrophy of the adrenal cortex in weanling rats which exhibit the syndrome of

hemorrhagic kidney. It is possible that this hypertrophy may contribute in some manner to the development of hypertension in animals surviving the syndrome. A normal amount of salt was present in the diets we employed, but further investigations along these lines are possibly indicated.

Calder (18a, 18b) has reported experiments in which a slight rise in the level of arterial pressure apparently occurred in rats which had been fed diets partially or completely deficient in the heat-

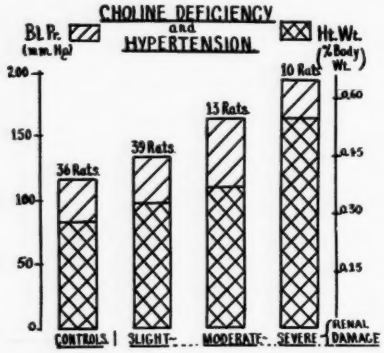


FIG. 5. COMPARISON OF HEART WEIGHTS AND SYSTOLIC-DIASTOLIC LEVELS of blood pressure (direct method of measurement) in the 4 groups of rats which totalled 98 in number. The upper limits of the bars indicate average levels of blood pressure for each group according to the scale on the left. The shorter, cross-hatched portion of each bar indicates for each group the average heart weight expressed as a percentage of body weight, according to the scale on the right. The number of rats in various groups is stated above each column. The animals were selected according to degree of parenchymal damage indicated at base of each column after assessment of microsections of kidneys.

stable fractions of the vitamin B complex. He found an average difference in blood pressure of 23 mm. Hg between the rats in his experimental and control groups, as determined by direct measurements taken by cannulation of the abdominal aorta. The levels of blood pressure of his experimental animals (direct readings) averaged only 144 mm. Hg. Calder did not report the cardiac weights of the animals in these experiments. Although he describes lesions of the renal parenchyma and thickening of both the arteries and arterioles with hyaline degeneration of the latter, his photomicrographs suggest that these changes were very slight. The parenchymal lesions resemble minor degrees of those which have been produced (8) in young adult rats maintained for several weeks on low-choline diets. As previously pointed out, Sobin and Landis (6) were unable to demonstrate alterations in the blood pressures of such

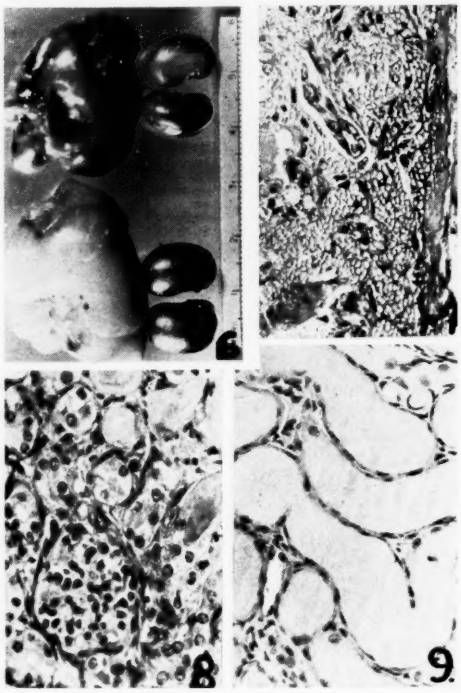


FIG. 6. APPEARANCE ON GROSS EXAMINATION of kidneys and liver (below) of a weanling rat fed the basal diet low in choline for a period of 7 days. Similar organs from a control rat pair-fed the basal diet supplemented with choline during the same period are included (above) for comparison. The scale at right is in centimeters. Note increase in size of these organs of the experimental animal, the pale appearance of its fatty liver and the dark hue of its kidneys which were dusky red.

FIG. 7. NECROTIC TUBULES IN SUBCAPSULAR REGION are widely separated by extravasated blood in this kidney section from a weanling rat fed a diet low in choline for 7 days. Hematoxylin and eosin stain. × 150.

FIG. 8. SWOLLEN TUBULES SURROUND A GLOMERULUS in the kidney of a weanling rat fed a diet low in choline for 5 days. Tubules further away from the glomerulus and nearer the periphery of the renal lobule (upper right) are necrotic. Hematoxylin and eosin stain. × 250.

FIG. 9. THE EPITHELIUM OF MEDULLARY TUBULES which are swollen with eosinophilic colloid is thinned and stretched. From the same section illustrated in preceding figure. × 250.

animals, and our investigations have confirmed theirs. The degree of hypertension reported by Clader is comparable perhaps to that which developed in the animals in our experiments which

comprised the group with only slight renal damage. Few of these animals were grouped as hypertensive, as in our experiments only those with levels of blood pressure (systolic-diastolic averages) of 150 mm Hg or more were so designated. If Calder's findings are confirmed and extended, it will require further work to decide whether or not choline deficiency is the responsible factor. In the meantime, it cannot be considered established that a significant hypertension can be produced by Calder's procedure.

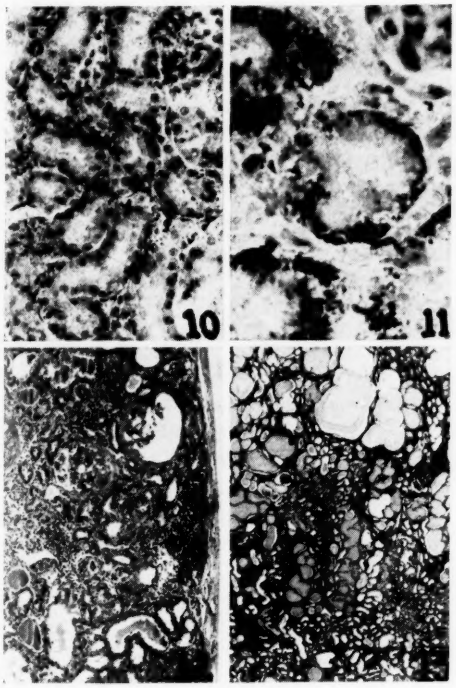


FIG. 10. THE LESION which is initially demonstrable in course of development of renal hemorrhagic syndrome of choline-deficient weanling rats consists of appearance of small sudanophilic droplets in epithelium of proximal convoluted tubules which become swollen and compress intervening capillaries. The fat droplets appear as small black dots in the photograph. Frozen section of renal cortex from a weanling rat fed the choline-deficient diet for 2 days; Sudan IV and hematoxylin; Wratten G and H filters; × 100.

FIG. 11. RELATIVELY LARGE AMOUNTS OF STAINABLE LIPID in tubules of renal cortex of a weanling rat fed the basal diet for 6 days. Frozen section; Sudan IV and hematoxylin; Wratten G and H filters; × 425.

FIG. 12. SEVERE PARENCHYMAL DAMAGE IN KIDNEY of a rat surviving the hemorrhagic renal syndrome and attaining maturity on a normal diet. Note glomerular fibrosis and subcapsular scarring. The capsule itself is slightly thickened. Hematoxylin and eosin stain. × 70.

FIG. 13. PARENCHYMAL DAMAGE IN KIDNEY of a rat in the same group as the animal from which the section illustrated in fig. 12 was prepared. In this case note extreme degree of tubular distension. Hematoxylin and eosin stain. × 25.

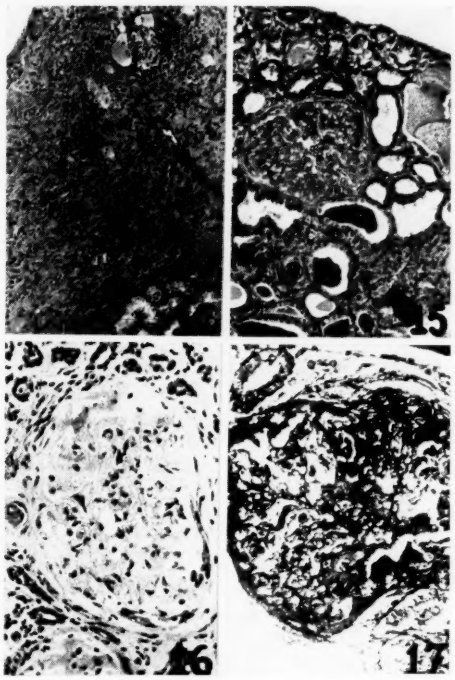


FIG. 14. EXTREME THICKENING OF RENAL CAPSULE of a rat which developed hypertension 2 months after kidney had been wrapped with gauze. A small area of underlying parenchyma is included in lower right of the field. For comparison with fig. 15. Hematoxylin and eosin stain; × 20.

FIG. 15. COMPLETE ABSENCE OF FIBROSIS OF RENAL CAPSULE of a rat developing hypertension subsequent to a brief period of dietary choline deficiency in early life. Note thickening of glomerular capsule, tubular distension and cellular hyperplasia of small artery in lower left of the field. Compare with preceding figure. Hematoxylin and eosin stain; × 50.

FIG. 16. HYALINIZATION OF A GLOMERULUS AND ARTERIOLE in kidney of a rat with hypertension subsequent to dietary choline deficiency. Gomori's chromium hematoxylin and phloxine stain; × 250.

FIG. 17. DIFFUSE THICKENING OF CAPILLARY LOOPS in a glomerulus of kidney of a rat with hypertension associated with choline deficiency in early life. Azocarmine, anilin blue and orange-G stain; Wratten B and E filters; × 250.

The method of producing experimental hypertension in rats by short periods of dietary choline deficiency in early life is one which may introduce less complications than do procedures such as injection of nephrotoxic sera, or those involving

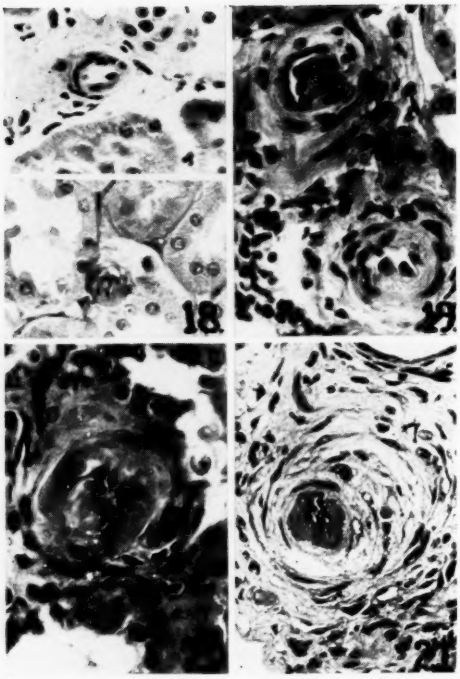


FIG. 18. EXAMPLES OF 2 NORMAL ARTERIOLES in renal cortices of kidneys of rats belonging to the *control* group. Compare with figs. 19, 20 and 21. Hematoxylin and eosin stain; × 300.

FIG. 19. EARLY CELLULAR HYPERPLASIA OF ARTERIOLES in kidney of a rat surviving the hemorrhagic renal syndrome with only a *slight* degree of parenchymal damage. This animal's blood pressure (systolic/diastolic average measured by a direct method) at the time it was killed was 150 mm. Hg. Hematoxylin and eosin stain; × 300.

FIG. 20. HYALINE DEGENERATION WITH A SUGGESTION OF EARLY NECROSIS in a cortical arteriole in kidney of rat surviving the hemorrhagic renal syndrome with a *moderate* degree of parenchymal damage. Blood pressure measured 170 mm. Hg (systolic/diastolic average). Hematoxylin and eosin stain; × 300.

FIG. 21. ADVANCED FIBRINOID NECROSIS OF AN ARTERIOLE in the renal cortex of a rat surviving a brief period of acute dietary choline deficiency in early life with a *severe* degree of parenchymal damage. Blood pressure measured 210 mm.Hg (systolic/diastolic average). Hematoxylin and eosin stain; × 300.

operations such as laparotomy. Theonly difference in the treatment of the animals of our control series and those in which hypertension was produced lay in the withholding of less than 0.02 grams of choline chloride from the diets of the latter for a brief period of 5 or 6 days.

Our findings and those of Sobin and Landis have indicated that if young rats which have survived the hemorrhagic renal syndrome are maintained on diets deficient in choline throughout the entire experimental period, hypertension will not be produced. If however, choline and its precursors (including protein) are restored to the diet after kidney damage has occurred, malignant hypertension may develop in later life. We have already suggested that an important factor in explaining the absence of hypertension in the first instance may be the slow rate of growth which occurs in this group of animals. The demands on the damaged cardiovascular system in these rats would be correspondingly lessened and renal metabolism would presumably be at a lower level because of the low percentage of protein in the deficient type of diet. The relative importance of these various factors in the etiology of the hypertension we have produced is a subject of investigations we are planning to pursue.

An important outcome of this work is the proof that extensive, and often fatal, pathological changes during adult life may have their originin a very short period of dietary abnormality in infancy. Correction of the abnormality during adult life, under certain conditions, may lead to pathological changes (hypertension in the present instance) which would otherwise not have occurred. These findings suggest that the later effects of lesions produced by other dietary deficiencies in very young animals may prove a fruitful field of investigation which may yield the investigator a rich harvest.

The nutritional aspects of this work have been made possible by a grant from the Nutrition Foundation, New York. The histological studies were supported by the National Research Council of Canada.

We are indebted to Dr. Jessie H. Ridout for a great deal of help with the diets. The technical assistance of D. H. Beaton and W. D. Wilson in the histological work is gratefully acknowledged.

REFERENCES

1. BEST, C. H., J. M. HERSHEY AND M. E. HUNTSMAN. *Am. J. Physiol.* 101: 7, 1932.
2. GRIFFITH, W. H. AND N. J. WADE. *J. Biol. Chem.* 131: 567, 1939.
3. GYÖRGY, P. AND H. GOLDBLATT. *J. Exper. Med.* 72: 1, 1940.
4. ENGEL, R. W. AND W. D. SALMON. *J. Nutrition* 22: 109, 1941.

5. HANDLER, P. *J. Nutrition* 31: 621, 1946.
6. SOBIN, S. S. AND E. M. LANDIS. *Am. J. Physiol.* 148: 557, 1947.
7. CHRISTENSEN, K. A. *Arch. Path.* 34: 633, 1942.
8. HARTROFT, W. S. *Brit. J. Exper. Path.* 29: 483, 1948.
9. SOBIN, S. S. *Am. J. Physiol.* 146: 179, 1946.
10. HANDLER, P. Personal communication to Professor C. H. Best, 1949.
11. PAGE, I. H. *Science* 89: 273, 1939.
12. CHANUTIN, A. AND E. B. FERRIS. *Arch. Int. Med.* 49: 767, 1932.
13. GOLDBLATT, H., J. LYNCH, R. F. HANZAL AND W. W. SUMMERVILLE. *J. Exper. Med.* 59: 347, 1934.
14. SELYE, H. *J. Em. Vet. Med. Assoc.* 103: 140, 1943.
15. KRARKOWER, C. A. AND H. E. HEINO. *Arch. Path.* 44: 143, 1947.
16. LENEL, R., L. N. KATZ AND S. RODBARD. *Am. J. Physiol.* 152: 556, 1948.
17. DEANE, H. W. AND R. E. OLSON. *Anat. Rec.* 100: 21, 1948.
18a. CALDER, R. M. *J. Exper. Med.* 76: 1, 1942.
18b. CALDER, R. M. *J. Exper. Med.* 79: 215, 1944.

American Association of Immunologists

Symposium on Mechanisms of Immunity to Viruses

Chairman: JOSEPH W. BEARD

DISTRIBUTION OF ANTIBODY TO POLIOMYELITIS IN VACCINATED AND PARALYTIC MONKEYS[1]

ISABEL M. MORGAN

From the Poliomyelitus Research Center, Department of Epidemiology, Johns Hopkins University

BALTIMORE, MARYLAND

THE study of immunity to poliomyelitis has taken on new interest since finding that, by intramuscular vaccination with active poliomyelitis virus, the majority of monkeys can be rendered immune to intracerebral challenge with large doses of active virus (1). This method has been effective in differentiating types of poliomyelitis virus (2, 3) as well as instrumental in studying immunogenesis of poliomyelitis. I plan to discuss the immune response to poliomyelitis in monkeys induced by a variety of exposures to virus.

The distribution of antibody in and out of the central nervous system in vaccinated animals has already been contrasted with that in paralyzed animals (4). These data will be reviewed and more recent observations will be presented on antibody distribution in vaccinated animals which have been challenged intracerebrally with various infectious and non-infectious nervous tissue suspensions.

Table 1 gives the distribution of antibody in fluids and CNS tissues of monkeys vaccinated with or paralyzed by Lansing virus.

The rhesus monkeys in the first group were vaccinated 4 to 6 times intramuscularly with 1 cc. doses of 10 per cent Lansing infected monkey spinal cord. At 6 weeks, spinal fluid and blood were taken. After perfusion with sterile saline solution, the monkeys were killed. The thalamus, cervical and lumbar enlargements of the spinal cord and the visual cortex were saved. The thalamus was of interest as the region of inoculation, and, like the anterior horn, a susceptible area. The visual cortex was selected as a non-susceptible area of gray matter. The spinal cord enlargements frozen on dry ice were quartered longitudinally and from the anterior quadrants the white matter was scraped away leaving sticks of gray matter. A single layer of gray matter was obtained from a flattened, frozen piece of visual cortex by scraping away the adjacent layers of white and gray matter. Suspensions of these tissues at a 1/3.2 dilution in saline were made in a TenBroeck tissue grinder. Further 10-fold dilutions were made from these. Each tissue and fluid was titrated by intracerebral mouse neutralization test, in the presence of 10 LD_{50} of Lansing virus.

From table 1 it can be seen that in 8 vaccinated monkeys, serum titers arranged in decreasing order ranged from high to moderate titers (titers of 3.0–3.8 are maximum in our experience). Yet no antibody was demonstrable in spinal fluid or the parts of the CNS saved. The titers recorded as <1 indicate that from 3 to 5 mice were spared of the group of 8 which received the 1/3.2 dilution of nervous tissue and virus. The sparing of a few mice is inadequate to establish either a true neutralization or a negative result and is therefore recorded as <1. Tests with many of these tissues which yielded borderline results have been repeated and again yielded an indefinite result.

The next group in table 1 of 11 monkeys which were paralyzed as a result of intracerebral injection of Lansing virus were also studied for antibody distribution in the same tissues (except for thalamus). A marked contrast to the vaccinated group is apparent. The highest titers appeared in

[1] Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

the anterior horn of these convalescents, killed from 16 days to 5 months after onset of paralysis. Serum titers approximated those of the spinal fluid (suggesting a spilling over of antibody from the affected regions of the CNS) whereas no antibody was demonstrable in the visual cortex. The levels in the anterior horn were in most instances 10- to 100-fold greater than those in the fluids.

TABLE 1. DISTRIBUTION OF ANTI-LANSING NEUTRALIZING ANTIBODY IN VACCINATED AND PARALYZED MONKEYS

	RHESUS NO.	SERUM	SPINAL FLUID	THALAMUS	ANTERIOR HORN	VISUAL CORTEX
Lansing Vaccinated	B726	2.8[1]	0		0	0
	B724	2.7	0		0	<1
	D926	2.6	0	0	0	
	B725	2.5	0		0	0
	D928	2.2	0	0	0	
	D927	2.1	0	<1	<1	
	B727	1.9	0		0	0
	D929	1.6	0	<1	0	
Lansing Paralyzed	B431	2.4	1.5+		2.9	0
	B429	2.2			3.1	0
	B739	1.9	1.9		2.9	0
	B736	1.6	1.0		2.6	0
	B737	1.2	1.0		2.3	0
	B444	1.2	1.5		3.3	0
	B738	<1	1.0		2.7	0
	B734	0	1.0		2.1	0
	B741	0	0		2.0	0
	B742	0	0		2.0	0
	B740	0	0		0	0
Brunhilde Paralyzed	D312				0	
	D374				0	
	D437				0	
	D438				0	

[1] Neg. log 50% endpoint by mouse neutralization test.

The last group of 4 individuals in table 1 were killed 3–7 weeks after onset of paralysis incited by Brunhilde virus. No antibody to Lansing virus was demonstrable in the anterior horn of the spinal cord. The Brunhilde strain of virus is known to be immunologically distinct from Lansing virus as shown by reciprocal immunity tests in vaccinated animals. However, a cross-relationship among poliomyelitis viruses is demonstrable by reinoculation of paralytic convalescent monkeys. Such animals are 100 per cent immune to the same virus as that with which they were vaccinated or to another poliomyelitis virus of the same immunological type. On the other hand, they are not fully susceptible to poliomyelitis virus of a different immunological type. For example, about half of a Brunhilde-convalescent group resists an intracerebral injection of a large dose of Lansing virus and vice versa (5). Just what is the basis of this relationship we do not know. The negative result presented here fails to throw light on the question.

TABLE 2. DISTRIBUTION OF NEUTRALIZING ANTIBODY IN LANSING-VACCINATED MONKEYS AFTER INTRACEREBRAL CHALLENGE

I.C. CHALLENGE	RHESUS NO.	OUTCOME	SERUM	SPINAL FLUID	THALAMUS	ANTERIOR HORN	VISUAL CORTEX
Lansing	D920	N	3.4[1]	2.5		2.0	
	E422	NP	3.3	1.5+	3.5+	1.5	1.3
	D921	N	3.0	2.3	3.0	2.0	
	E423	N	2.6	0.5	1.9	1.1	1.0
	D922	N	2.3	<1.0	1.0	0	
	E425	N	2.2	1.5+	2.2	0	1.5
Lansing, Minnesota	D210	P	3.4			3.4	
	D218	P	3.3			1.9	
	D221	P	3.2			2.5	
	D220	P	2.9			1.5	
	D217	P	2.7			2.0	
Minnesota	D924	P	2.5	1.4	2.8	2.9	
	D925	P	2.4	1.6	2.0	2.2	
	D923	P	<1	0	<1	0	
Normal CNS	E433	N	2.7	0.	0	1.2	<1
	E430	N	2.6	0	0	0	0
	E431	N	2.4	0	<1	0	0
	E428	N	1.9	0	<1	0	<1
	E429	N	1.8	0	0	0	<1

N = no paralysis. NP = non-paralytic poliomyelitis. P = paralysis.

[1] Neg. log 50% endpoint by mouse neutralization test.

Having found that in vaccinated animals antibody is found primarily in the blood stream, and that with sufficiently high level of antibody such individuals are immune to intracereberal injection of large doses of homologous virus, I was interested to find what the distribution of antibody would be after such intracerebral challenge. Accordingly, 6 Lansing-vaccinated monkeys which proved immune to intracerebral injection of 10,000 LD_{50} of Lansing virus were bled, tapped for spinal fluid, perfused with sterile saline and killed for study.

They appear as the first group in table 2. It

can be seen that they had moderate to high levels of serum antibody. The majority had antibody in the spinal fluid at a somewhat lower level. Within the CNS, antibody levels of the thalamus approximated those of the serum, but what is more remarkable was the finding of antibody in the majority of individuals in distant parts of the CNS, viz., the anterior horn and visual cortex. (No antibody was demonstrable in the anterior horn of the 2 individuals with the lowest serum antibody levels.) This finding immediately raises the question of whether the virus incited infection. In three individuals (E422 series) the spinal cord was examined histologically at levels T_2 and L_1 and one (E422) was found to have had inapparent infection. However, the pattern of antibody in this individual was no different from that in the others (with the possible exception of an outstanding concentration in the thalamus). In a larger series of 23 monkeys, vaccinated with and immune to intracerebral injection of Lansing virus, from which the medulla and spinal cord at levels C7 and L7 were examined, this individual alone showed evidence of infection. Thus the immunity of the vaccinated animal as a rule does not depend on CNS infection. It is fascinating to speculate on what may account for this antibody response at remote parts of the CNS. Either it is simply an immune response to introduction of antigen, perhaps as a secondary stimulus, or possibly the antigen as active virus exerts some immunogenic effect beyond what an inactivated preparation would, in spite of no histological evidence of infection. Against the latter hypothesis is the fact that antibody response seems to be universal through the CNS, and not confined to the susceptible areas. The effect of injection of inactivated virus intracerebrally has not been studied. Another control has been done, however, on the effect of intracerebral injection of normal CNS tissue suspension in vaccinated animals. This group of 5 monkeys appears at the bottom of table 2. With moderately high serum antibody levels, they had no antibody definitely demonstrable in the CNS, with the possible exception of the anterior horn of one individual (no material was available to repeat this test). This control serves to establish that the injection of CNS tissue (with the hemorrhage and ensuing local inflammatory reaction) does not in itself introduce anti-poliomyelitic neutralizing substance into the CNS.

Another line of attack is the effect of heterotypic poliomyelitic virus infection in Lansing-vaccinated animals. This approach was thought of in connection with the question of whether the antibody in the affected areas of CNS in a Lansing paralysed animal was developed locally or represented an accummulation of antibody produced elsewhere. The second and third groups of animals in table 2 bear on this point. The monkeys in both groups were vaccinated intramuscularly with Lansing virus, and challenged subsequently with Minnesota virus. In one group, they received an intervening Lansing challenge. In the group which failed to have this homologous challenge, one individual (D923) had such low titer of circulating antibody that it probably would not have passed such a challenge and is therefore not comparable to the others. The other 7 individuals showed moderate to high levels of antibody in the anterior horn of the spinal cord and in this regard are comparable to Lansing paralytic animals. The two individuals in which thalamus was tested showed equally high levels of antibody there, with lower levels in the spinal fluid. These findings support the contention that antibody may accumulate in the CNS in response to an inflammation reaction. This is perhaps opposed to the hypothesis which I have proposed of local formation of antibody in the affected parts of the CNS. There is one factor, however, which must be looked into further, and that is the question of possible antigenic relationship of these two poliomyelitis viruses. We now know they are sufficiently distinct immunologically to be considered two types, but to what degree there is a relationship we have not yet sufficient quantitative data to know. This problem recalls the resistance of approximately 50 per cent paralytic convalescent animals to reinfection with heterotypic virus. But as yet there is no immunolgical evidence to account for this resistance.

To summarize, Lansing paralytic convalescent monkeys have highest antibody levels in the affected parts of the CNS, in contrast to vaccinated animals in which antibody is demonstrable in the serum but not in the CNS. If circulating antibody is sufficiently high in such vaccinated individuals, they are immune to intracerebral injection of a large dose of homologous virus. In response to such injection of virus, antibody appears throughout the nervous system, even in distant, insusceptible areas.

Monkeys paralyzed with one poliomyelitis virus show no demonstrable antibody in the anterior horn of the spinal cord to a heterotypic virus. Nevertheless, monkeys vaccinated with one virus

and paralyzed with a heterotypic poliomyelitis virus acquire in the anterior horn high levels of neutralizing antibody to the virus with which they were vaccinated.

Does this suggest that antigenic stimulus with any poliomyelitis virus within the CNS in an animal already vaccinated with a poliomyelits virus renders the entire CNS immune? Since this stimulus has not yet been given in the form of inactivated virus, it is all too obvious how many steps will have to be taken before dreaming of any practical application of such an idea to man.

REFERENCES

1. MORGAN, I. M., H. A. HOWE AND D. BODIAN. *Am. J. Hyg.* 45: 379, 1947.
2. MORGAN, I. M. *Am. J. Hyg.* 49: 225, 1949.
3. BODIAN, D., I. M. MORGAN AND H. A. HOWE. *Am. J. Hyg.* 49: 234, 1949.
4. MORGAN, I. M. *Am. J. Hyg.* 45: 390, 1947.
5. BODIAN, D. *Am. J. Hyg.* 49: 200, 1949.

LOCAL IMMUNE RESPONSE AND VIRAL GROWTH RATE AS FACTORS IN IMMUNITY TO EQUINE ENCEPHALOMYELITIS VIRUS

R. WALTER SCHLESINGER

From the Division of Infectious Diseases, The Public Health Research Institute of the City of New York, Inc.

NEW YORK CITY

THIS discussion will be limited to a brief review of recent studies on the mechanism of acquired active immunity of mice to intracerebral challenge doses of equine encephalomyelitis (E.E.) virus (1, 2). These studies were the outgrowth of earlier work on the possible significance of humoral factors, specifically neutralizing antibody, in relation to resistance to infection.

Previous efforts to establish or rule out the existence of a quantitative correlation between antibody titers and resistance to infection with E.E. or other viruses were limited, by and large, to the antibody present at the time of exposure to the infecting dose of virus. This approach has yielded inconsistent or inconclusive results. It was suggested that the relatively high titers of neutralizing antibody in the serum, which appeared to be associated with resistance to intracerebral challenge doses of E.E. virus (3, 4), reflected the presence within the central nervous system of antibody in adequate concentration. This suggestion was based on the finding (4, 5) that antibody in actively or passively immunized animals was distributed between serum and the central nervous system at a ratio of 100/1 to 300/1. Two observations made it clear, however, that such a concept of immunity as a static function of serum antibody titer was untenable: *a*) In immunized guinea pigs which had survived an intracerebral challenge dose the serum/brain ratio was altered as a result of a disproportionate increase in neutralizing titer of brain tissue (6). *b*) The degree of resistance of mice vaccinated with formalinized Western E.E. virus was found to vary with the use of different strains of this virus as challenge inoculum (7). In particular, mice which following vaccination were resistant to certain strains were not or only partially protected against the 'R.I.' strain. This was true even for mice immunized with vaccines prepared from the R.I. strain itself. Since it was not possible to show serological differences between the R.I. and the other strains, it was of interest to investigate the nature of this phenomenon.

One point of distinction between the R.I. and the other strains was that the former had undergone numerous brain-to-brain passages in mice. In the course of this procedure it had acquired the ability to kill mice in about one half of the time required by comparable amounts of the less well adapted strains.

Comparison of the rates of multiplication in the mouse brain of the 'fast' R.I. strain and a representative 'slow' strain (Kelser) has revealed differences corresponding to their relative rapidity of action. The belief that increase in the rate of viral multiplication is responsible for the difference in response of immunized mice to the R.I. and the Kelser strains has been strengthened by the finding that the latter, after 37 additional rapid brain-to-brain passages, simulated the R.I. strain in rapidity of action, and that vaccinated mice were significantly more resistant to the 'slow' parent strain than to its 'fast' derivative.

The difference in response to the two strains was not demonstrable in mice vaccinated with *large* doses of inactivated virus. By reducing the dosage of vaccine, a level of immunity could be induced at which all of the mice challenged with 10^5 LD_{50} of the 'slow' strain would survive, while those challenged with the same amount of the 'fast' strain would succumb.

When the fate of the two virus strains in the brains of mice so treated was studied, the following difference was found: the R.I. strain multiplied at about the same rate in vaccinated as in normal mice. The Kelser strain, to which the vaccinated mice were immune, also multiplied, but at a slower rate than in normal mice. The maxi-

mum increase was about 100-fold over the amount found at 3 hours after inoculation, and virus persisted at the higher level for as long as 4 days after inoculation. No virus was demonstrable under these conditions in brains harvested on or after the 5th day.

Further tests were predicated on the assumption that a local immune response, stimulated by the challenge inoculum, could compete successfully with the slowly multiplying strain but not with the fast R.I. strain. It was found that brains in which virus was no longer detectable (i.e., in the experiment just described, those harvested from the 5th day on) were capable of neutralizing virus. The neutralizing titer of brain tissue increased markedly, reaching a maximum level after about 10 days and maintaining it for at least 4 months. As a result, the serum/brain ratio of neutralizing titers was reduced from a 'physiological' value of about 200/1 to less than 10/1.

The magnitude of this shift in serum/brain ratio depended inversely on the degree of prechallenge immunity. One may assume that intensive vaccination by itself stimulated a primary immune response sufficient to render part of the intracerebral inoculum inert as antigen. This fits in with the observation already mentioned that the difference in response to challenge with 'slow' and 'fast' strains was not demonstrable in mice vaccinated with large doses of inactive virus.

The view that the increase in neutralizing titer of brain tissue is due to the antigenic booster action of the intracerebral inoculum finds indirect support in the observation that mice vaccinated with the smallest immunogenic amounts of vaccine were protected against *large* amounts, as much as 10^5 LD_{50}, but only partially resistant to about 10 to 100 LD_{50} of the 'slow' strain. Comparative studies on the rate of viral multiplication after intracerebral inoculation of varying amounts have yielded converging curves for large seed inocula and more closely parallel curves for small amounts. This has been interpreted as indicating that after inoculation of large amounts only a fraction of the inoculum is utilized in the initiation of infection, leaving the bulk free to act as antigen. Small inocula, on the other hand, are presumably taken up quantitatively by susceptible cells, leaving no virus available as free antigen. Hence, while in animals 'sensitized' by extremely low-grade immunization there is no adequate primary immune response to overcome small challenge doses, large doses exert an anamnestic booster effect resulting in the animal's survival. This type of 'paradoxical response' has also been described for other infections, notably in the case of rabies virus where standard potency tests on vaccines involve intracerebral challenge inoculations in immunized mice (8, 9). It may be responsible for misleading results, especially when the challenge test does not include the entire range of effective virus dilutions.

The conclusion that the virus-inactivating substance present in brain tissue is neutralizing antibody is based on the following evidence: *a*) The reduction in serum/brain ratio of neutralizing titers following challenge is paralleled by similar changes in the ratio of titers of complement-fixing antibody. *b*) It is specific. In animals vaccinated with a mixture of inactive Western *and* Eastern E.E. viruses and challenged with active virus of either type, the relative concentration of the homologous, but not of the heterologous, antibody in brain tissue rises. *c*) Like antibody in the serum, it follows the 'percentage law' on dilution of underneutralized mixtures.

It may be said, then, that the fate of a vaccinated animal after intracerebral challenge inoculation of E.E. virus depends on a variety of factors. Death or *complete* protection are not the only alternatives. Even in the immune, surviving animal, the challenge virus may multiply and persist at high levels for several days. Except for hyperimmunized mice, the decisive factor appears to be the more effective force in a competition between rate of viral multiplication and intensity of local immune response. The latter, in turn, is determined not only by the degree of 'sensitization' acquired with vaccination but also by the size of the challenge inoculum. The term 'sensitization' is used in this connection as denoting an increase in the potential ability of the immunized animal to respond locally to the antigenic stimulus of the intracerebral inoculum. It is chiefly this increase by which mice vaccinated with relatively small doses of inactive virus differ from normal animals.

It is not intended to suggest that mechanisms as complex as the one postulated here apply to other virus-host systems. It must be remembered that the artefact of an intracerebral inoculation is associated with extensive trauma in the immediate vicinity of susceptible cells. The inoculum, moreover, presumably is diffused throughout the closed system of the central nervous system. Thus, if it contains a large excess of virus which

does not participate in initiating infection, this excess is free to act as antigen wherever cellular elements involved in the immune response are exposed to it. It is conceivable that the mechanism of immunity may not involve all of the factors mentioned to the same extent in those infections, for example, in which the virus acts directly on exposed surface cells or in which the site of inoculation is removed from the susceptible tissue.

REFERENCES

1. Schlesinger, R. W. *J. Exper. Med.* 89: 491, 1949.
2. Schlesinger, R. W. *J. Exper. Med.* 89: 507, 1949.
3. Morgan, I. M. and P. K. Olitsky. *J. Immunol.* 42: 445, 1941.
4. Morgan, I. M., R. W. Schlesinger, and P. K. Olitsky. *J. Exper. Med.* 76: 357, 1942.
5. Freund, J. *J. Exper. Med.* 51: 889, 1930. (Also personal communication.)
6. Schlesinger, R. W., P. K. Olitsky, and I. M. Morgan. *J. Exper. Med.* 80: 197, 1944.
7. Olitsky, P. K., I. M. Morgan, and R. W. Schlesinger. *Proc. Soc. Exper. Biol. & Med.* 59: 93, 1945.
8. Habel, K. and J. T. Wright. *Pub. Health Rep. U.S.P.H.S.* 63: 44, 1948.
9. Casals, J. Personal communication.

REMARKS ON CELLULAR RESISTANCE TO MAMMALIAN VIRUSES[1]

JOHN F. ENDERS

From the Harvard Medical School and The Children's Medical Center

BOSTON, MASSACHUSETTS

INTEREST arose in the theory of cellular immunity as applied to viral infections when it became apparent that this class of pathogens could multiply only in close association with living cells—indeed probably only within the confines of the cell membrane itself. With the establishment of this fact the obvious deduction was drawn that these pathogens were particularly fitted to elicit an immune response dependent exclusively upon the development of intracellular antiviral factors. This inference has led to numerous experimental attempts to obtain evidence of acquired cellular immunity to viruses. To a few of the more significant of these I shall refer toward the end of this paper. It may be stated at once, however, that they have not provided an unequivocal answer to the problem. At present, the available evidence permits merely the assessment of the probability that this phenomenon may occur.

In contrast to the doubtful status of acquired cellular immunity, the evidence for the existence of natural or innate resistance of cells to infection by viruses is convincing. I shall therefore review this evidence in some detail, especially that which has been gained through studies in tissue cultures. Before doing so, I shall define the concept of cellular resistance which I have followed in the selection of the material.

Cellular resistance depends upon an intrinsic or acquired property or properties of the cell, which independent of factors in the body fluids may prevent the multiplication of a virus or destroy its activity. Obviously this definition is extremely broad and would include such non-specific and non-immunologic properties as an impermeable cell membrane or the absence of an enzyme system. To embrace, however, the phenomena of natural immunity to virsuses such breadth of definition is clearly essential.

[1] Presented as part of the Symposium on Mechanisms of Immunity to Viruses, Detroit, Mich., April 20, 1949.

NATURAL OR INNATE CELLULAR RESISTANCE

Factors other than the activity of cells which might be responsible for natural resistance. At the outset we may appropriately consider the possibility that certain non-cellular factors could be responsible for the natural resistance of certain species. To do so will serve to reveal the dominant position of the cell in this type of immunity. Such factors may be grouped under three headings: *1*) non-specific antiviral effects of plasma, or other body fluids which are destroyed by moderate heating; *2*) thermostabile virus-neutralizing substances; *3*) physical conditions incompatible with survival or multiplication of the virus.

In respect to the first of these, data exist which indicate clearly that fresh unheated serum from normal susceptible animals may inactivate or restrict the multiplication of a virus if mixed with it and then inoculated. For example, Douglas and Smith (1) found that fresh rabbit serum mixed with vaccinia virus and then inoculated intradermally into rabbits definitely modified the development of the lesion. We have also observed, in unpublished experiments, that fresh rabbit and cat sera added to small amounts of vaccinia virus reduce the size and number of pocks which subsequently develop on the chorioallantoic membrane of chick embryos. Recently Leymaster and Ward (2) in a study of the augmenting effect of complement on mumps neutralizing antibody found that undiluted or slightly diluted unheated sera of normal monkeys and presumably susceptible human beings were capable of preventing infection of chick embryos by the mumps virus.

Although such observations are worthy of note for those engaged in the demonstration and titration of virus neutralizing antibodies, one cannot regard the antiviral effect *in vitro* of unheated normal serum as playing a significant part in natural resistance. For it is obvious, as in the case of anthrax, that there is no correlation be-

tween host susceptibility and the capacity of the serum *in vitro* to inhibit the virus.

A purely physical factor, the normal temperature of the body, may render some species naturally resistant to attack by certain viruses. Dr. Pearson and I (3) a few years ago obtained evidence that the striking difference between the high susceptibility of the chick embryo and the complete resistance of the newly hatched chick to infection with the Melbourne strain of influenza A virus was adequately accounted for by the difference in temperature of the two hosts. Subsequently McLean and his co-workers (4) likewise found that the multiplication of a strain of influenza B virus was markedly inhibited at temperatures corresponding to the body temperature of birds. I am not aware that similar studies have been made with other viruses such as those causing equine encephalomyelitis which are highly virulent for the chick embryo but to which the fowl is almost or completely resistant. Such investigations, though, would be of value in assessing the general importance of a high body temperature in the natural resistance of the fowl to many mammalian viruses as compared with that of its embryo.

In addition to these three classes of non-cellular factors, one can conjecture that other conditions such as an unusually high hydrogen-ion concentration of the saliva or the presence of antagonistic micro-organisms in the flora of the nose, the throat or the gastrointestinal tract might arrest or destroy certain viruses at their portal of entry. In general. however, it would seem, that with the exception of differences in normal body temperature, non-cellular factors are of little significance.

Evidence that cellular resistance is the principal factor in natural immunity. By exclusion, then, one arrives at the tissue cells as the major factor in natural resistance to viruses. Moreover, a great deal of evidence—far too much to be reviewed here—of a more direct character has been accumulated through studies of the behavior of viruses in living animals especially in regard to the type of cell which is injured and the organ or tissue in which multiplication occurs. From certain points of view even more convincing than these observations made *in vivo* are the results obtained by tissue culture techniques. Since I have been interested for some time in these methods and since the time is limited, I shall confine my remarks mainly to a review of the results so obtained in respect to the problems under discussion. In their evaluation, however, one should bear in mind, that, as Beard and Rous (5) have said, the physiological isolation inevitable to all cultivation *in vitro* is not always an advantage from the experimental point of view.

When directed to the investigation of natural cellular immunity the tissue culture technique has shown that in most cases in which adult tissues have been employed the growth or failure of growth of a given virus *in vitro* is correlated with susceptibility or resistance *in vivo*. So far, I believe, lack of such correlation has been observed only when the resistance *in vitro* of embryonic tissues has been compared with that of the living adult of the same or another species. In these instances where embryonic tissues have proved susceptible and the living adult relatively resistant, it is possible that differences in the properties of the cells may be involved. But before this conclusion can be accepted, the effect of non-cellular factors such as a more elevated body temperature of the adult must be eliminated.

Examples of correlation between natural resistance *in vivo* and *in vitro* have been described by Andrewes (6) and by Ivanovics and Hyde (7) in the case of Virus III infections of rabbits. The same relationship has been demonstrated by Andrewes (8) for the salivary gland virus of guinea pigs. Hallauer (9) who has reviewed the literature up to 1939 cites experiments of Frenkel and van Waveren who found that the virus of foot and mouth disease can only be cultivated in the tissues of susceptible species such as the cow, sheep and the guinea pig. Findlay (10) was able to obtain multiplication of fowl pox only in tissues of susceptible birds. A particularly good instance of agreement between host resistance and the *in vitro* behavior of the cells is to be found in the studies of Hallauer (9) on different strains of fowl plague virus. He was unable to propagate in mouse embryonic tissues a strain to which mice were entirely refractory, although he succeeded easily when he used chick embryonic tissue. On the other hand, working with two strains which induced infection in mice as well as in fowl, he was able to cultivate both agents in cultures of mouse tissue as well as in those composed of chicken cells. Sabin and Olitsky (12) succeeded in growing a strain of human poliomyelitis only in cultures consisting of human embryonic brain

fragments. The virus rapidly disappeared from cultures of chicken or rabbit nervous tissues. Employing the Lansing strain of poliomyelitis virus we have recently confirmed the findings of Sabin and Olitsky in respect to growth in human nervous tissues (13). As yet, however, we have been unable to obtain evidence of the multiplication of this virus in fragments of mouse brain. Since the Lansing strain is pathogenic for mice, this unexpected finding might appear as an exception to the general correlation between *in vivo* and *in vitro* susceptibility. But there may well be other reasons for this discrepancy.

The difference in resistance to a number of viruses of various types of cells derived from the same host species has also been investigated by means of tissue cultures. Unfortunately there have been few studies in which pure cultures of cells have been employed. They are sufficient, however, to establish the fact that some viruses can multiply only in certain cell types. Thus Carrel (14) as early as 1926 showed that the Rous sarcoma virus failed to proliferate in cultures of chick fibroblasts but actively increased in cultures of monocytes. The agents of foot and mouth disease and fowl plague according to Köbe and Fertig (15) and to Hallauer (11) multiply only in epithelium. Findings of this sort serve to demonstrate even more clearly than in the living animal the strict cytotropism of some viruses. They also forcefully reveal how closely adapted these viruses are to subtle differences in metabolism or other properties which must distinguish one cell type from another. In contrast to such agents the cytotropism of certain other viruses appears to be much broader. The virus of vaccinia has been grown in pure cultures of Kupffer cells by Beard and Rous (5) and in cultures of blood monocytes by Florman and Enders (16) as well as in corneal epithelium by Rivers and his co-workers (17).

With Feller and Weller (18) we have obtained evidence that this virus will also increase in fibroblasts although pure cell cultures were not used. If cell injury and death be criteria for the multiplication of a virus, the agent of equine encephalomyelitis is pancytotropic in respect to the cells of the chick embryo as revealed by unpublished experiments of Relova and myself and by the more recent observations of Huang (19). In experiments carried out during the past year with the Lansing strain of poliomyelitis virus we have, in collaboration with Robbins and Weller (13), determined that multiplication takes place in cultures not only of nervous tissue but in those composed of fragments of intestine as well as in those consisting mainly of skin and striated muscle. Since no intact nerve cells were present in the cultures of skin and muscle it is highly probable that cells other than those of the nervous system are susceptible to this strain of the virus.

Varying degree of resistance of cells to different viruses. We have so far been mainly concerned with observations which show that certain cells may be completely resistant while others are susceptible. There are many data to suggest, however,that between these two extremes different types of cells may exhibit variable degrees of susceptibility to different viruses. Variation in susceptibility may be expressed in two ways: first, by differences in the quantity of the virus produced by different cells within a fixed period; second, by the extent of the injury the cell undergoes as a result of the presence of the virus.

That different tissues, at least, vary in their capacity to support the multiplication of a virus is indicated by numerous experiments in which the virus content of tissues removed from infected animals has been measured. As illustrations, it will be sufficient to mention two investigations in which determinations of the concentration of virus present in different parts of the infected chick embryo have been carried out. Pearson (20) employing the Melbourne strain of influenza A virus found it present in largest quantities in the allantoic fluid and yolk sac. Relatively low concentrations were observed in the amniotic fluid and in the embryo itself. Similarly in unpublished experiments we have observed that with an egg-adapted strain of mumps virus the highest yields were obtained in the embryonic fluids and membranes. In the embryo itself small amounts were encountered. Very little has been done with tissue cultures to determine whether isolated cells vary in their capacity to support the growth of virus. I can only refer to some observations made in my laboratory a few years ago when it was noted that in roller tube cultures of fibroblasts developing from fragments of chick embryonic heart muscle the production of vaccinia virus appeared to be somewhat greater than in comparable cultures of blood monocytes.

As I have said, the extent of injury the cell undergoes as a result of the presence of a virus may also be interpreted as measure of its resistance or susceptibility to the pathogenic effect of the infecting agent. Thus it has long been apparent that the nerve cells are particularly sus-

ceptible to certain neurotropic viruses, the parenchyma cells of the liver to yellow fever virus and so on. Indeed so little affected are other types of cells in such diseases that it has often been held that the responsible agents multiply only in those cells which exhibit gross manifestations of injury. Evidence obtained by a variety of techniques during the past 15 or 20 years shows, however, that obvious cell injury is not the inevitable consequence of virus multiplication. Some of the most conclusive information on this important point has been gained through measurements of virus increase in tissue cultures correlated with the histological examination of the infected tissues. Thus the virus of equine encephalomyelitis not only increases very rapidly in cultures of mixed chicken embryonic tissues but within 24 to 48 hours brings about the death of all or nearly all the cells as indicated by morphological appearance and cessation of metabolism (19). In sharp contrast the virus of mumps, as we have recently found, increases relatively slowly in cultures of chick amniotic membrane and continues to multiply for several weeks without inducing any recognizable change in cell morphology or metabolism as measured by acid production (21). Similarly Florman and I (16) could detect no pathologic changes in cultures of blood monocytes in which vaccinia virus continued to multiply for several weeks. Vaccinia virus, however, induces the formation of cytoplasmic inclusions in fibroblasts growing out of fragments of chick embryonic tissues in roller tube cultures (18). Nevertheless the death of these cells does not appear to ensue immediately. Indeed such cultures may continue to metabolize and cell growth take place for many weeks while multiplication of the virus continues at a fairly constant rate. It is also probable that certain other viruses exert an injurious effect on cells only after prolonged contact. Thus we have lately repeatedly observed in cultures of various human embryonic tissues infected with the Lansing strain of poliomyelitis virus degenerative changes in most of the cells after 25 to 32 days of cultivation although it was shown that the agent had undergone active multiplication at a much earlier period when no cytologic abnormalities attributable to the effect of the virus could be discerned (13). Dr. Herbert Morgan has observed an analogous series of events in tissue cultures infected with psittacosis virus (22).

I have dwelt at this length upon the variation in the pathogenicity of various viruses for different cell types not only because it illustrates a difference in resistance between cells but to reemphasize the fact that the multiplication of some mammalian viruses may occur, and often does occur, quite independently of any demonstrable injurious effect on the cells with which they are associated. This fact, as we shall see, may be important in considering the problem of acquired cellular immunity which I shall now very briefly discuss.

PROBLEM OF ACQUIRED CELLULAR IMMUNITY TO VIRUSES

Indications that cells acquire specific resistance as a result of infection. The possibility that tissue cells may acquire a specific immunity to viruses is suggested mainly by the failure in numerous instances to demonstrate a constant correlation between the presence or concentration of virus-neutralizing antibodies in the body fluids and the resistance to reinoculation of an animal that has been previously infected or vaccinated.

Many examples of this phenomenon could be cited. It will suffice here to recall only one. Hodes and Webster (23) found that mice which had been vaccinated with St. Louis encephalitis virus developed a very considerable resistance to reinfection which lasted for 6 or 7 weeks. During most of this period, however, virus neutralizing antibody could not be demonstrated in the serum. Later when such antibody had appeared, resistance to reinfection had diminished. At the end of 20 weeks when the animals again became entirely susceptible to infection, the antibody was still present in high titer in the blood.

Findings of this sort are indeed difficult to explain entirely on the grounds of humoral immunity. In some cases as Vieuchange and Galli (24) have shown in dermal infections with vaccinia virus the neutralizing antibody first emerges locally in the area of the infected tissues and only appears later in the blood stream. But the interval of time between these two events is usually short, not exceeding a few days. It is difficult therefore to explain such phenomena as described by Webster and Hodes on the basis of local antibody retention.

The theory of course has long been current that under certain circumstances the antibody produced at the site of infection remains permanently fixed to the cells and does not enter the circulation in appreciable quantities. Lépine (25), just before the war, again discussed this hypothesis and presented some evidence to support it. On the whole,

though, in spite of many attempts to demonstrate antibodies exclusively associated with the cells no convincing evidence that this occurs has been presented.

In other instances interference between the inactive virus originally introduced and that employed for reinoculation might be responsible for the observed resistance. The effects of interference, however, would not be expected to be very durable—at least when inactivated virus is employed as an immunizing agent.

Failure to demonstrate the development of cellular mechanisms which prevent the multiplication of virus. If, then, neither virus neutralizing antibody nor the interference phenomenon adequately explain such manifestations of acquired resistance, can it be shown that the cells of immunized animals removed from the influence of neutralizing antibody or other inhibiting factors in the serum fail to support the multiplication of the homologous virus?

The bulk of experimental results would seem to leave little doubt that the answer to this question is in the negative. When adequate precautions have been taken to eliminate absorbed antibody, fixed tissue cells derived from immune animals have been shown by the tissue culture method to be as susceptible as those obtained from normal individuals. Moreover, various types of phagocytic cells removed from immune donors when freed of antibody by repeated washing have not been found to inhibit the multiplication of virus to a degree exceeding that exhibited by comparable cells from normal animals. In support of these statements it is only necessary to refer to the work of Andrewes on the propagation of herpes simplex virus (26) and Virus III (27) in fragments of rabbit testicle, the experiments of Rous, Hudack and MacMaster (28) with rabbit embryonic tissues and vaccinia virus, those of Beard and Rous (5) with isolated Kuppfer cells and vaccinia and those of Smorodinseff and Shishkina (29) with polymorphonuclear leucocytes and macrophages from mice immunized against influenza A virus.

Possibly unrecognized mechanisms dependent upon increased resistance of cells. In conclusion we shall inquire whether there may be any other possible mechanisms of acquired immunity to viruses associated with or developed by the cells. Relatively recent observations suggest that perhaps two hitherto unrecognized responses of this sort may occur. Various workers such as Grabar and Schoen (30) in France as cited by Lépine (25) and Gard (31) in Sweden have obtained from extracts of infected tissue removed during the acute stage of certain virus diseases, factors which when mixed with the homologous virus inhibit its activity. Whether or not these antiviral factors are to be identified with serum antibodies or whether they are distinct products of the cell or possibly the virus itself has not yet been conclusively determined. It is probable, however, that they do not represent the usual type of antibody. In a personal communication Dr. Gard has informed me that the factor he has obtained from mouse brain infected with Theiler's virus is more heat resistant than the known antibodies and also resists digestion by such active proteolytic enzymes as ficin. Grabar and Schoen prepared their material from the brains and lungs of mice infected with the virus of lymphogranuloma venereum. Mice infected with this virus do not develop in the blood appreciable quantities of neutralizing antibodies. It is unlikely therefore, that the antiviral activity of these extracts can be attributed to the usual type of neutralizing antibody. In this connection the recent experiments of Bodian (32) are also relevant. He has shown that monkeys suffering from acute poliomyelitis caused by one serologic type of virus are usually refractory to intracerebral inoculation with a heterologous strain. In contrast, convalescent monkeys are not immune to heterologous strains. Bodian does not believe that the resistance exhibited by monkeys in the acute phase of the disease depends upon the interference phenomenon.

The possibility, then, that under the impact of primary infections certain cells may respond by the production of antiviral factors which have hitherto not been recognized must obviously be further investigated.

Another approach to the problem may have been opened by observations by Galli and Cipollone (33) which were reported in 1941, but have not yet been confirmed. Following repeated unilateral intratesticular inoculation of formalin-inactivated vaccinia virus in rabbits, these workers noted that a high concentration of virus neutralizing antibodies developed in the blood serum. In such animals, in spite of the presence of the antibody, active virus introduced into the skin or into the untreated testis produced reactions which were only moderately or slightly less severe than those observed in unvaccinated rabbits. In contrast, the injection of the same quantity of

virus into the vaccinated testicle did not induce an inflammatory reaction. Yet titration of the virus content of each testicle showed that the agent had increased greatly and to the same degree in both. Failure of the vaccinated testicle to react could be demonstrated at least 37 days after inoculation of the last dose of vaccine. If these experiments can be confirmed, it would seem that resistance to the pathogenic effect of a virus may be induced without increasing the capacity of the cell to prevent the multiplication of the agent. The thought that the response of a cell may be altered artificially in this manner, is perhaps rendered more acceptable by the fact that in normal cells the response to injury is not an inevitable consequence of virus proliferation.

REFERENCES

1. Douglas, S. R. and W. Smith. *Brit. J. Exper. Path.* 11: 96, 1930.
2. Leymaster, G. R. and T. G. Ward. *J. Immunol.* 61: 95, 1949.
3. Enders, J. F. and H. E. Pearson, *Proc. Soc. Exper. Biol. & Med.* 48: 143, 1941.
4. McLean, I. W., Jr., D. Beard, A. R. Taylor, D. G. Sharp, J. W. Beard, A. E. Feller, and J. H. Dingle. *J. Immunol.* 48: 305, 1944.
5. Beard, J. W. and P. Rous. *J. Exper. Med.* 67: 883, 1938.
6. Andrewes, C. H. *Brit. J. Exper. Path.* 10: 188, 1929.
7. Ivanovics, G. and R. R. Hyde, *Am. J. Hyg.* 23: 55, 1936.
8. Andrewes, C. H. *Brit. J. Exper. Path.* 11: 23, 1930.
9. Hallauer, C. *Handbuch der Virusforschung*, Erste Hälfte, Dritter Abschnitt. J. Springer. Wien, 1938, p. 368.
10. Findlay, G. M. *Brit. J. Exper. Path.* 9: 28, 1928.
11. Hallauer, C. *Zt. F. Hygiene.* 113: 61, 1931.
12. Sabin, A. B. and P. K. Olitsky. *Proc. Soc. Exper. Biol. & Med.* 34: 357, 1936.
13. Enders, J. F., T. H. Weller, and F. C. Robbins. *Science* 109: 85, 1949.
14. Carrel, A. *J. Exper. Med.* 43: 647, 1926.
15. Köbe, K. and H. Fertig. *Z. Bakt.* Abt. I, orig. 138: 14, 1937.
16. Florman, A. L. and J. F. Enders. *J. Immunol.* 43: 159, 1942.
17. Rivers, T. M., E. Haagen, and R. S. Muckenfuss. *J. Exper. Med.* 50: 665, 1929.
18. Feller, A. E., J. F. Enders, and T. H. Weller. *J. Exper. Med.* 72: 367, 1940.
19. Huang, C. H. *J. Exper. Med.* 78: 111, 1943.
20. Pearson, H. E. *J. Bact.* 48: 369, 1944.
21. Weller, T. H. and J. F. Enders. *Proc. Soc. Exper. Biol. & Med.* 69: 124, 1948; also unpublished observations.
22. Morgan, H. R. Personal communication.
23. Hodes, H. L. and L. T. Webster. *J. Exper. Med.* 68: 263, 1938.
24. Vieuchange, J. and F. Galli. *C. R. Acad. Sci.* (Paris) 208: 2031, 1939.
25. Lépine, P. *Arch. gesamte Virusforsch.* 2: 406, 1942.
26. Andrewes, C. H. *J. Path. & Bact.* 33: 301, 1930.
27. Andrewes, C. H. *Brit. J. Exper. Path.* 10: 273, 1929.
28. Rous, P., P. D. McMaster, and S. S. Hudack. *J. Exper. Med.* 61: 657, 1935.
29. Smorodintseff, A. A. and O. I. Shishkina. *Arch. gesamte Virusforsch.* 2: 175, 1941.
30. Grabar, P. and R. Schoen. *Bull. soc. chim. biol.* 23: 281, 1941.
31. Gard, S. Personal communication.
32. Bodian, D. *Am. J. Hyg.* 49: 200, 1949.
33. Galli, F. and R. Cipollone. *Arch. gesamte Virusforsch.* 1: 350, 1941.

INHIBITION OF SOME HEMAGGLUTINATING VIRUSES

S. G. ANDERSON

From the Department of Preventive Medicine, Western Reserve University

CLEVELAND, OHIO

THE polysaccharides have been studied more intensively than several other agents (1–3) which are known to inhibit viruses. This study of the polysaccharides has been integrated with research on the relation of virus to host cell. I will first briefly mention this relation as it applies to the viruses of the mumps-influenza group, and later will describe inhibitors of these viruses found in serum, ovarian cyst contents and other mucous secretions, allantoic fluid, vegetable polysaccharides and certain bacterial extracts.

You are all familiar with the phenomenon of the attachment of the hemagglutinating viruses to red cells (4), and know that with many strains there is a subsequent elution of apparently unchanged virus and a permanent alteration of the cell. Elution is believed to be due to enzymic alteration of cellular receptors.

Several types of bacteria, notably the *cholera vibrio*, produce soluble products which cause receptor alteration much more rapidly and completely than do the viruses of mumps, Newcastle disease or influenza (5).

The attachment of virus to red cell has been considered to be a model of the early stages of the infection of living cells. The comparison may be only partly valid (6).

Polysaccharide inhibitors may be conceived to act in two ways: they may prevent the attachment of virus to cells (red cells, or living or dead tissue cells) or may modify the multiplication of virus within living cells.

In normal animal serum, there are at least two types of inhibitor. One is possibly a globulin; it is destroyed by heat at 65° C. in less than 30 minutes (7). It is slowly destroyed by cholera filtrates, but apparently is not altered by incubation with live virus. It inhibits hemagglutination by living virus, but only to low titer; this titer varies between strains, being in general higher with the more recently isolated strains. The titer also varies considerably according to the particular chicken which supplies the red cells for the Hirst inhibition test (8).

A second class of inhibitor we believe to be a mucoid (9) and I will assume this to be so during the rest of this paper. It inhibits hemagglutination by virus which has been heated to 56° C. for 30 minutes and it is the substance responsible for the phenomenon described by Francis (10) in 1947. It is thermostable; in fact the apparent titer of the substance increases up to 8-fold when a 1:10 dilution of serum in saline is heated to 100° C. for one minute. It is rapidly destroyed by cholera filtrates, and also by live virus at 37° C. (11). In the latter case the system reaches an equilibrium, at which the amount of inhibitor inactivated is characteristic of the virus used (12). The inhibitor is destroyed by large amounts of periodate (12), a reagent which is particularly active against carbohydrates.

A similar mucoid inhibitor has been isolated from human ovarian cysts (12), and the action of periodate on this mucoid has been particularly studied (13). The raw mucoid will inhibit hemagglutination by heated WSE influenza virus, but not by living WSE, and will reduce the infective titer of WSE 10–100 fold. A large amount of periodate will destroy this inhibitory power completely, but mucoid treated with small amounts of periodate will inhibit hemagglutination by living WSE virus, and will reduce the infectivity of WSE about 1000-fold as tested in the chick embryo. You will see that mild periodate treatment of mucoid has magnified its inhibitory power against live virus; such treated mucoid can no longer be destroyed by virus, or by cholera enzyme.

Essentially the same properties are shared by inhibitors from a wide variety of sources, such as egg white (14), allantoic fluid (15, 16) and aqueous extracts of many tissues (17), particularly of certain salivary glands of ferrets, men, mice and sheep. It is possible that blood group A substance should be included in this list (12). A similar inhibitor can be extracted from red cells of certain species (17, 18); partly because of this, there has been commonly accepted the working hypothesis that red cell receptors and soluble inhibitors are closely related.

Probably all these inhibitors act by competing with the red cell for the virus surface. It seems likely that this competition gives rise to a state of dynamic equilibrium between the various possible combinations of virus, cell and inhibitor. For example the estimated titer of either inhibitor in serum is greatly influenced by the type of chicken cells used in the system (11). When cells with a low affinity for heated virus are employed, the titer of mucoid inhibitor in a serum may be, say 5000; if cells with a high affinity are added to the system, the inhibitory titer will be in the region of 100 with the same serum. This suggests that the union of virus with inhibitor is reversible, and that, in the circumstances I have described, the dissociation of virus and inhibitor is not due primarily to enzymic action of the virus. This is supported by the finding of Hardy and Horsfall (19) that virus in the presence of a certain inhibitor in allantoic fluid is equally as infectious as virus in the absence of inhibitor, though the virus cannot free itself from this inhibitor by enzymic action.

We have seen that in this competition for the virus, the advantage is given to the mucoid rather than to the red cells, either when the mucoid is partially treated with periodate or when the virus is heated. Either procedure prevents subsequent action of the virus enzyme. It has been concluded that these mucoids act as inhibitors because their attraction for the heated virus is not reduced by enzymic activity, and conversely that live virus is not inhibited because it can inactivate the inhibitor (19–21). There appear to be two difficulties to this explanation: *a*) Although at 0° C. enzymic action of virus on mucoid inhibitor is minimal, live Lee virus in our hands is not inhibited to significant titer even at this temperature. *b*) When live virus agglutinates red cells at 0° C. in the presence of inhibitor, the thermostable agent which can be recovered from the system is still highly potent in the inhibition of heated virus. Of course, there can be no doubt that live virus will react with inhibitor and destroy it at 37° C. and that heated virus cannot react in this way. But is it safe to assume that this functional difference is the basis of the inhibition of heated virus by mucoid? It may be, for example, that the greater effect of the mucoid inhibitor on heated virus is due to a thermal alteration of the adsorptive surface of the virus rather than to the loss of enzymic activity per se (22).

Svedmyr reports (16) that the inhibitory agent for PR8 hemagglutinin, present in normal allantoic fluid, is in the main connected with particles with a sedimentation constant of 200 S. This component reacts in precipitation tests with virus, and does so in definite proportions. The inhibitor progressively decreases in amount in infected embryos, and is destroyed *in vitro* by live virus and by cholera filtrate. It does not inhibit virus multiplication in the egg. Svedmyr briefly describes a second type of hemagglutinin inhibitor in allantoic fluid, an inhibitor which is not susceptible to inactivation by virus and which is not sedimentable at 27,000 r.p.m. These two inhibitors may well correspond to the two described before as being present in normal serum. Hardy and Horsfall (19) have suggested that in infected allantoic fluid some of the virus is linked to an inhibitor which it does not inactivate. It may be that this substance is the same as Svedmyr's unsedimentable inhibitor and the same as the thermolabile inhibitor in normal serum described by McCrea in 1946 (7).

Svedmyr believes the thermostable particulate inhibitor may be derived directly from the cell receptors lining the allantoic cavity. This concept of the identity of the 'soluble' thermostable inhibitor, the receptors on red cells, and the receptors on susceptible tissue cells, constantly appears. On the basis of the latter part of this hypothesis, Dr. Stone (23) in 1947 carried out a series of interesting experiments. She reasoned that if the hypothesis were correct, the cholera filtrate which readily destroyed red cell receptors should also destroy tissue cell receptors. With their receptors destroyed, the cells would not be susceptible to virus attack. Her experiments confirmed this. Prior treatment with cholera filtrate in the allantoic cavity of chick embryos prevented subsequent infection in all the eggs inoculated with up to 30 infective doses of CAM influenza virus, and in about 90 per cent of eggs inoculated with 50 ID_{50} of NDV. The protective effect lasted for about two days following the cholera treatment. After about 54 hours the receptors were apparently regenerating and the cells again became susceptible.

You will remember that the salivary mucus of ferret, mouse and man had a particularly high titer of thermostable inhibitor; in these species clinical signs of influenza are readily produced. There is a suggestion, though no more, that the sheep, which is the only other animal known to have a high titer of inhibitor, may also be clini-

cally infected. These correlations seem more than coincidental, but their significance remains open to question. It may be that the presence of inhibitor in the respiratory secretions merely reflects the presence of virus receptors on respiratory cells; or the relation may be more direct in that virus more readily persists, and infects in the presence of a mucin to which it can be adsorbed and with which it can react enzymically. It is cogent to ask whether in other species the respiratory mucin, which is not so susceptible to virus action, prevents the rapid spread of virus over the respiratory cells.

Bacterial polysaccharides were reported by Levine and Frisch in 1932 (24) to inhibit the lysis of the corresponding bacteria by bacteriophage. Burnet and Gough (25, 26) confirmed the specificity of this inhibition and believed that it was due to the blocking of the bacteriophage by the polysaccharide. More recently, Maurer and Woolley (27) described the non-specific action of pectin in preventing lysis of bacteria after they have been infected with bacteriophage. Apple pectin has also been found by Green and Woolley (28, 29) to prevent hemagglutination due to Lee virus, and in doing so to adhere both to virus and to red cells. It was also partially or completely effective in inhibiting Lee virus multiplication in the fertile hen egg, even when given 1–2 hours after inoculation of the virus. The authors were stimulated to investigate apple pectin because it was thought to be possibly an analogue of the hypothetical cellular substrate of the influenza virus. Subsequently Woolley (30) has reviewed the subject of competition between analogues and has suggested (31) that such competition may be the basis of the inhibitory action of apple pectin.

It is not known whether the effect of these pectins is destroyed by virus or by cholera filtrates. Woolley (29) states that of the polysaccharides tested the most active were polygalacturonides but that those containing glucuronic acid and mannuronic acid had some potency.

So far I have referred to competitive adsorption and the prevention of receptor-virus union as the mechanism of inhibition. The point of view of Horsfall and Ginsberg (32) strikes a new note. They believe that bacterial polysaccharides inhibit virus multiplication by an intracellular action. They believe that the virus can enter the cell but cannot multiply there because of the presence of the intracellular polysaccharide. The background of their thesis is briefly this:

Horsfall and McCarty (33) reported that substances, probably polysaccharides, found in several species of bacteria would inhibit multiplication of pneumonia virus of mice in the mouse lung. This effect was seen even if the polysaccharide was given up to 4 days after the initial infection. Ginsberg and Horsfall (32) later found that 1 mg. of Friedlander bacillus polysaccharide would prevent the reproduction of mumps virus in the allantoic cavity of chick embryos so long as the inoculum was less than 10^3 ID_{50}. These authors placed polysaccharide, together with a large amount of virus in the allantoic cavity, and found some virus was absorbed. They assumed that if a smaller inoculum were used (such as 10^3 ID_{50}), a portion of that, too, would be absorbed. They believed therefore that polysaccharide inhibits mumps inside, not outside the cell.

To summarize, I have described two types of inhibitors of hemagglutination in serum. One is destroyed at 65° C. and is possibly protein; the other is thermostable and is probably mucoid. Both act by combining reversibly with extracellular virus. Two inhibitors described in allantoic fluid, and the inhibitor found in mucous secretions, may be of the same type as those in serum. Certain bacterial polysaccharides have been postulated to inhibit intracellular virus multiplication.

Work in this field is a step toward unravelling the relation between virus and host cell. If it is true that the mucoid or vegetable polysaccharide inhibitors are analogues of the cell receptor, we have one phase of that relation reproduced in a simple system and in solution. On the other hand, as Horsfall, McCarty and Ginsberg have said, their work may open the way to a study of *intracellular* metabolic processes. Because of this I believe it is very important to have confirmation of their hypothesis that bacterial polysaccharide affects intracellular virus metabolism rather than adsorption of virus on cell.

REFERENCES

1. Green, R. H., A. F. Rasmussen, Jr. and J. E. Smadel. *Pub. Health Rep.* 61: 1401, 1946.
2. Green, R. H. *Proc. Soc. Exper. Biol. & Med.* 67: 483, 1948.
3. Wiseman, R. W., G. Meiklejohn, D. B. Lackman, C. Wagner and G. W. Beveridge. *J. Immunol.* 54: 9, 1946.

4. Hirst, G. K. *J. Exper. Med.* 75: 49, 1942.
5. Stone, J. D. *Australian J. Exper. Biol. & M. Sc.* 25: 137, 1947.
6. Florman, A. L. *J. Bact.* 55: 183, 1948.
7. McCrea, J. F. *Australian J. Exper. Biol. & M. Sc.* 24: 283, 1946.
8. Anderson, S. G., F. M. Burnet and J. D. Stone. *Australian J. Exper. Biol. & M. Sc.* 24: 269, 1946.
9. McCrea, J. F. *Australian J. Exper. Biol. & M. Sc.* 26: 355, 1948.
10. Francis, T., Jr. *J. Exper. Med.* 85: 1, 1947.
11. Anderson, S. G. *Australian J. Exper. Biol. & M. Sc.* 26: 347, 1948.
12. Burnet, F. M. *Australian J. Exper. Biol. M. Sc.* 26: 371, 1948.
13. Burnet, F. M. *Australian J. Exper. Biol. & M. Sc.* 26: 381, 1948.
14. Lanni, F. and J. W. Beard. *Proc. Soc. Exper. Biol. & Med.* 68: 312, 1948.
15. Beveridge, W. and P. E. Lind. *Australian Exper. Biol. & M. Sc.* 24: 127, 1946.
16. Svedmyr, A. *Brit. J. Exper. Path.* 29: 295, 309, 1948.
17. Friedewald, W. F., E. S. Miller and L. R. Whatley. *J. Exper. Med.* 86: 65, 1947.
18. Bovarnick, M. and P. M. deBurgh. *Science* 105: 550, 1947.
19. Hardy, P. H. and F. L. Horsfall. *J. Exper. Med.* 88: 463, 1948.
20. Burnet, F. M. *Lancet* 1: 7, 1948.
21. Hirst, G. K. *J. Exper. Med.* 87: 301, 1948.
22. Anderson, S. G., F. M. Burnet, S. F. de St. Groth, J. F. McCrea and J. D. Stone. *Australian J. Exper. Biol.* 26: 403, 1948.
23. Stone, J. D. *Nature* 159: 780, 1947.
24. Levine, P. and A. W. Frisch. *Proc. Soc. Exper. Biol. & Med.* 30: 993, 1932; 31: 46, 1933.
25. Burnet, F. M. *J. Path. & Bact.* 38: 285, 1934.
26. Gough, G. A. C. and F. M. Burnet. *J. Path. & Bact.* 38: 301, 1934.
27. Maurer, F. D. and D. W. Woolley. *Proc. Soc. Exper. Biol. & Med.* 67: 379, 1948.
28. Green, R. H. and D. W. Woolley. *J. Exper. Med.* 86: 55, 1947.
29. Woolley, D. W. and R. H. Green. *J. Bact.* 54: 63, 1947.
30. Woolley, D. W. *Physiol. Rev.* 27: 308, 1947.
31. Woolley, D. W. *J. Exper. Med.* 89: 11, 1948.
32. Ginsberg, H. and F. L. Horsfall. *J. Exper. Med.* 87: 385, 411, 1948.
33. Horsfall, F. and M. McCarty. *J. Exper. Med.* 85: 623, 1947.

MECHANISMS OF IMMUNITY TO VIRUSES: THE INTERFERENCE PHENOMENON[1]

WERNER HENLE

From the Children's Hospital of Philadelphia, Department of Pediatrics, University of Pennsylvania School of Medicine

PHILADELPHIA, PENNSYLVANIA

In a discussion of immunity or resistance to virus diseases, some consideration must be given to the interference phenomenon. It is by now a well-known fact that a host organism, be it bacterium, plant or animal, when infected with one virus frequently fails to support propagation of another viral agent. Such interference phenomena were first noted between antigenically closely related agents which differed in certain of their biological properties, such as their tissue tropisms or their adaptation to various hosts. However, subsequently interference was also observed between antigenically distinct agents. In some of these instances, the viruses nevertheless must be considered biologically closely related, as in the case of interference between influenza A and B viruses, or between the agents of eastern and western equine encephalomyelitis. In other instances, such relationships are not readily apparent. In any case, these observations show clearly that the interference phenomenon is distinct from specific immunity as mediated by antibodies.

The interference phenomenon has aroused great interest on account of its theoretical and possibly practical implications. Consequently, a great deal of effort has been expended on the study of this phenomenon and the resulting information has become so extensive that it is impossible to review it at this time. I should like to limit my discussion, therefore, to interference between inactivated and active viruses—an observation first recorded in the bacteriophage field by Luria and Delbrück, since under such conditions, many factors appear to be eliminated which possibly could complicate study and interpretation of interference between two fully active viruses. And, in this limited field, I should like to single out a few questions concerning the interference between inactive and active influenza viruses in the allantoic cavity of the chick embryo which still appear quite problematic, whereas other more clearly established facts will only briefly be mentioned.

There is little doubt that the interfering agent in this system is identical with the virus particle. There exists a rough correlation between the active virus titer before irradiation by ultraviolet light, and the degree of interference obtained after inactivation. The interfering capacity is sedimented in the centrifuge at speeds required for the sedimentation of the active virus. It is removed from suspension by filtration and adsorbed onto and eluted from chicken red cells to an extent comparable to that observed with the active virus.

Thus it seems quite definite that the interfering property is closely linked with the virus particle. However, a marked degree of differentiation within the virus particle becomes apparent in considering the effect of ultraviolet irradiation upon the various properties of influenza virus. These are destroyed in the following order: *1*) The capacity to propagate; *2*) the toxic activity; *3*) the interfering property; *4*) the hemagglutination phenomenon including the elution mechanism and, at the same time, the complement-fixing activity due to the virus antigen; and *5*) the complement-fixing activity due to the soluble antigen.

Recently, some evidence has been presented which seems to indicate that not only the fully developed virus particle will cause interference but that at some stage of the developmental cycle, virus material is obtained which is non-infectious as yet, but already endowed with the interfering property. Gard and von Magnus showed that upon serial passage of undiluted allantoic fluids, two types of particles may appear in the sub-culture as determined by analytical centrifugation. One of these possesses all the attributes of influenza virus and, therefore, is identical with the infectious principle, whereas the other, smaller one, which has not been isolated as yet, according to all indications is non-infectious but causes hemagglutination and possibly interference. This latter particle is considered by these

[1] The studies described in this paper were done under a grant-in-aid from the U. S. Public Health Service.

authors as precursor or immature form of the virus.

A similar suggestion could serve as an explanation of the following data. If one injects a large dose of optimally prepared virus into the allantoic sac and studies thereafter, at hourly intervals, the allantoic fluids and membranes from representative numbers of eggs, for their infectivity, hemagglutinating and complement-fixing activities, it can be seen, allowing one hour for adsorption of the seed, that the infectivity in the fluid and tissue remains constant for 6 and 5 hours, respectively. On the other hand, development of hemagglutinins becomes measurable after the third hour, and they increase 30- to 100-fold before any rise in infectivity is noted. The complement-fixing antigen develops in the membranes even before the hemagglutinins, a fact recently reported also by Hoyle. This series of events is the more striking if one considers that the order of increasing sensitivity of these three tests is just the reverse, the complement fixation test being the least sensitive.

Further experiments revealed that the rise in hemagglutinins, prior to that of infectivity, is accompanied by the development of interfering activity as measured after inactivation of the various preparations by ultraviolet light. Although the origin of this non-infectious, but hemagglutinating, interfering and complement-fixing material is not definitely established, whether it is a breakdown product of the seed virus or an intermediary stage of virus formation, all indications point to the latter possibility at present.

As far as the interactions between host and interfering agent are concerned, the interference phenomenon can be induced only by direct contact of the host cells with the irradiated virus. The effect can not be counteracted either by flushing of the allantoic cavity with saline solution, or by the injection of potent immune serum immediately after the administration of the interfering agent. It is apparent, therefore, that interference is induced very rapidly, and possibly within less than one minute. The cells thus altered remain so for at least 6 days. It does not seem to matter whether a few or 10 million ID_{50} of active virus are used for the challenge, the results of interference appear indentical. However, recent observations seem to indicate that the interfering activity may be overcome to some extent by the use of excessive amounts of active virus, a point which is at present under investigation.

The quantitative aspects of the interference phenomenon in this system have not been clearly worked out as yet. Rough estimates of the number of cells lining the allantoic cavity of the 11- to 12-day-old chick embryo by two different approaches led to the figure of about 10^8 cells. The equivalent of approximately 10^8 ID_{50} of virus in the inactivated state is required to produce interference to such an extent that no measurable amounts of hemagglutinin are formed. Thus it appears that the equivalent of about one ID_{50} per cell will cause interference. However, it is not known whether every cell lining the allantoic sac will support viral propagation; and the number of virus particles constituting one ID_{50}, likewise, has not been definitely determined although it appears to be of the order of 10 and possibly less. These considerations would imply that the quantitative aspects are very similar to those encountered in the bacteriophage system by Luria and Delbrück.

The interference reaction is non-specific in the sense that inactivated influenza virus interferes with the active agents, not only of the homologous but also of the heterologous types, as well as with some strains of mumps virus, equine encephalomyelitis, epidemic keratoconjunctivitis, Newcastle disease, and possibly other viruses. There is no obvious difference in the degree of cross-interference between influenza A and B viruses, if the irradiated virus is injected prior to the administration of the active agents. However, if the irradiated virus is injected after infection, during the first hour of the first infectious cycle, then the homologous irradiated virus may markedly affect viral propagation in the tissues, whereas heterologous irradiated virus inhibits only to a lesser extent. This would seem to indicate that interference may be obtained at two different levels of the infectious cycle; at the first, a relatively non-specific effect is obtained, whereas at the second it appears rather specific for the type.

Various hypotheses have been proposed to furnish an explanation for the interference reaction. Many of these can be disregarded since they obviously do not apply to the system under study. However, two of these, suggested by Ziegler and Horsfall, and by Delbrück and Luria, respectively, require discussion. They are *1*) blockade or destruction by the primarily injected virus of cell receptors which mediate also infection of the host tissue by the second virus, and *2*) blockade of a key enzyme in the host cell by the interfering

agent which is necessary also for the propagation of the excluded virus.

The cell receptor theory, of course, is based on the studies of the hemagglutination phenomenon. As is well known, influenza virus is adsorbed onto and subsequently eluted from chicken red cells, and the erythrocytes thus treated become non-agglutinable, as measured by the addition of fresh homologous virus and, to a varying extent, by other strains of virus of the homologous and heterologous types. Similar phenomena of adsorption and elution have been observed by Hirst and by Stone, to occur in the lungs of ferrets and mice, and in the allantoic cavity of the chick embryo, under conditions which in all probability, would have precluded viral multiplication. Elution of active or inactivated virus has not been demonstrated as yet in the intact host. Furthermore, as has been pointed out previously, ultraviolet irradiation of influenza viruses destroyed the interfering property more rapidly than the hemagglutinating property. In other words, on prolonged irradiation a preparation may be obtained which does not cause interference but still is adsorbed and eluted from red cells to an apparently unaltered degree.

Finally, cells which have been treated with interfering preparations of irradiated virus still may adsorb additional virus of the homologous or heterologous types.

Although receptor blockade thus can be ruled out as the major cause of interference, it has not been entirely excluded as a contributing factor. Adsorption of virus onto the allantoic tissue amounts to only about 70 per cent in the average, the remainder of the seed virus being left free in the allantoic fluid. If this equilibrium is markedly disturbed by secondary injection of large amounts of virus, the additional adsorption apparently is more pronounced than in the case of small amounts of challenge virus, where less additional adsorption seems to take place. These relationships will bear further clarification since the experimental conditions are such as to approach closely the limits of accuracy of the available technics used for the assays.

As to the key enzyme hypothesis of Delbrück and Luria it seems to fit best to most of the available data. It is possible that the enzyme or enzymes affected are necessary both for viral and host cell multiplication. It was found that bacteria, treated with irradiated phage, discontinued multiplication, and, similarly, the allantoic sac of the 8-day-old chick embryo, injected with irradiated influenza virus, failed to grow thereafter at the normal rate. If the key enzyme hypothesis is correct, one would have to postulate now that one enzyme system must be involved in propagation of a number of viruses, and, consequently, its blockade would be responsible for the interference phenomenon between immunologically distinct viruses. The specific interference obtained by injection of irradiated virus of the homologous type within the first hour after infection, i.e., during the first infectious cycle, would seem to have to be caused by blockade of another enzyme system involved in production of components needed only for formation of virus of the homologous type.

It is obvious from these remarks that we are still far from having a satisfactory explanation for the interference phenomenon and, indeed, it may very well be that not a single explanation will ultimately fit all the observed facts.